1877

THE ROLE OF *ACTINOMYCES VISCOSUS* IN DENTAL PLAQUE.

J.S. VAN DER HOEVEN

THE ROLE OF *ACTINOMYCES VISCOSUS* IN DENTAL PLAQUE. ECOLOGICAL AND BIOCHEMICAL ASPECTS

DE ROL VAN *ACTINOMYCES VISCOSUS* IN DE TANDPLAQUE. EKOLOGISCHE EN BIOCHEMISCHE ASPEKTEN

PROMOTORES:

DR. K.G. KÖNIG
hoogleraar preventieve en sociale tandheelkunde

DR. IR. G.D. VOGELS
lector mikrobiologie

THE ROLE OF *ACTINOMYCES VISCOSUS* IN DENTAL PLAQUE. ECOLOGICAL AND BIOCHEMICAL ASPECTS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN
DE RECTOR MAGNIFICUS PROF. MR. F.J.F.M. DUYNSTEE,
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN OP
VRIJDAG 27 SEPTEMBER 1974
DES NAMIDDAGS OM 3 UUR PRECIES

door

JOHANNES SIMON VAN DER HOEVEN
geboren te Zwanenburg

Druk: Offsetdrukkerij Fakulteit der Wiskunde en Natuurwetenschappen
Nijmegen

Aan Anneke en onze zonen Tycho en Rutger

Uit het Instituut voor Preventieve en Sociale Tandheelkunde, Fakulteit der Geneeskunde van de Katholieke Universiteit te Nijmegen.

De heren Jan Lukassen en Jan Koedam dank ik voor het uitstekend biotechnisch werk, vooral op het moeilijke terrein van de gnotobiose De dames Mariette van den Hof en Elly Lurvink en de heer Henk Franken dank ik voor hun bekwame biochemische en mikrobiologische assistentie bij de experimenten. Mejuffrouw Joke Bolder ben ik bijzonder dankbaar voor het typen van de manuscripten en het uitvoeren van vele korrekties De heren H C M Reckers, J L.M van de Kamp, H.W.A Bongaarts en L J.H. Hofman dank ik voor hun vele goede diensten De heer Fons Plasschaert dank ik voor het statistisch werk en vele kritische bijdragen. De heer Jaap Maltha dank ik voor het histologisch werk en zijn stimulerende belangstelling

I would like to thank professor Bernard Guggenheim (Zurich) for his original idea that was worked out in this thesis
I would like to expresss my gratitude to Mr. George Bowden (London) for his excellent contributions.
Furthermore I should like to expres my appreciation to S Karger, Basel, Publishers, for their kind permission to reprint the text of chapters II, III and IV

Last but not least bedank ik de heer Frans Mikx, bladeren door dit proefschrift maakt duidelijk waarom

N.B. Promotores mogen op deze plaats niet worden bedankt, artikel 23 promotiereglement.

Contents

Samenvatting

Omtrent de mondflora en het ontstaan van caries werden aan het eind van de vorige eeuw inzichten geformuleerd die tot op heden hun geldigheid hebben behouden.

Miller (1889) toonde aan dat tal van mikro-organismen in de mondholte in staat zijn koolhydraten tot zuur te vergisten. Zijn chemo-parasitaire caries-theorie die hierop was gebaseerd vond algemeen ingang. Black (1898) en Williams (1897) richtten de aandacht op de bakteriemassa die voorkomt op het tandoppervlak.
Black gebruikte in dit verband het eerst de term plaque.
Williams gaf aan dat de bakteriemassa een diffusiehindernis vormt waardoor lokaal geproduceerd zuur niet onmiddellijk door het speeksel kan worden verdund en geneutraliseerd, en dientengevolge inwerkt op de tand.

Het is opvallend dat in de jaren hierna de interpretatie van Williams nauwelijks stimulerend heeft gewerkt op het verloop van het verder onderzoek. Pas in de tweede helft van deze eeuw is het onderzoek naar de tandplaque goed op gang gekomen. Voor die tijd hield men zich voornamelijk bezig met bakteriën uit het speeksel.

De waarneming dat talrijke soorten mikro-organismen naast elkaar in de plaque aanwezig zijn heeft aanleiding gegeven tot de veronderstelling dat wisselwerkingen tussen bakteriën van groot belang zijn voor de stofwisseling en daarmee het effekt van de tandplaque. Ekologisch onderzoek hiernaar is tot op heden nauwelijks verricht.

De vraag in hoeverre mikrobiële interakties in de tandplaque van invloed zijn op de cariogeniteit werd door König en Guggenheim (1965) voor het eerst experimenteel benaderd. Zij vonden dat de cariesaktiviteit in ratten die waren beënt met *Strep. mutans* sterk afhankelijk was van de samenstelling van de rest van de mikroflora wanneer die werd beïnvloed met behulp van een antibiotikum. Deze waarneming leidde tot een interessante overweging: er vanuitgaande dat de cariogene aanvalskracht van het ene mikro-organisme kan worden afgeslagen door een ander is het misschien mogelijk om in de mond een niet cariogene mikroflora te vestigen.
Met dit thema op de achtergrond werd in ons laboratorium het ekosysteem van de tandplaque in een reeks experimenten bestudeerd.

In hoofdstuk II van dit proefschrift wordt de cariogeniteit van *Strep. mutans* in mono-geassocieerde ratten vergeleken met die in ratten die boven-

dien met een SPF* mikroflora waren geassocieerd. De wisselwerking van *Strep. mutans* met de SPF-mikroflora leidt tot verlaging van de cariogeniteit.

Aangezien er te weinig bekend is van de SPF mikroflora kan de aard van de wisselwerking onmogelijk worden aangegeven. De waargenomen cariesreduktie zou bovendien uitsluitend het gevolg kunnen zijn van de "verdunning" van het cariogene mikro-organisme *Strep. mutans* door de SPF-mikroflora. Daarom is het noodzakelijk bij het bestuderen van mikrobiële interakties in de plaque van een eenvoudiger model uit te gaan. Voor dit doel komt het gnotobiotisch diermodel in aanmerking.

Bij de vorming van de tandplaque spelen extracellulaire bakteriële polymeren een belangrijke rol (zie b.v. het overzichtsartikel van Guggenheim 1971). In dit verband werd veel onderzoek verricht naar de synthese van extracellulaire polysaccharide door *Strep. mutans* en *Strep. sanguis*, beide mikro-organismen, die een voorkeur vertonen voor groei op het tandoppervlak. Deze streptokokken zijn in staat saccharose om te zetten tot polysaccharide. Hierbij zijn twee types saccharose-specifieke enzymen betrokken, de glukosyl - en de fruktosyl transferases, die respektievelijk glukanen (dextraan en mutaan) en fruktanen (levaan) maken. Vanwege deze omzetting krijgt saccharose een bijzondere plaats in de reeks van koolhydraten die voorkomen in de menselijke voeding.

In hoofdstuk III wordt de vraag gesteld in hoeverre de rol van saccharose uniek is. d.w.z. of polysaccharide synthese in de plaque volstrekt afhankelijk is van de aanwezigheid van saccharose. Men kan zich voorstellen dat ook in afwezigheid van saccharose bepaalde mikro-organismen in staat zijn extracellulair polysaccharide te synthetiseren en op die manier de plaquevorming te bevorderen. De beantwoording van deze vraag heeft ook praktische betekenis omdat ze te maken heeft met het sukses dat men kan verwachten van de vervanging van saccharose door een ander zoetsmakend koolhydraat in de voeding. Het blijkt dat het verstrekken van een glukoserijk dieet aan ratten leidt tot een sterke vermeerdering van een slijm producerend mikro-organisme, *Actynomyces viscosus* in de plaque, terwijl een saccharose dieet de groei van extracellulair polysaccharide producerende streptokokken bevordert. Dit resultaat bevestigt de betekenis van de voeding voor de mikrobiële samenstelling van de tandplaque.

Het extracellulair slijm van *A. viscosus* dat uit een bouillon kultuur wordt verkregen door neerslaan met alkohol, is samengesteld uit een aantal

* SPF = specific pathogen free

komponenten die duidelijk verschillen in chemische samenstelling. De belangrijkste komponent bestaat in hoofdzaak uit glukosamine, waarschijnlijk in de geacetyleerde vorm. Het aantonen van het extracellulair materiaal van *A. viscosus* in de tandplaque zal moeilijk zijn omdat tal van andere komponenten, zoals speekseleiwitten en bakteriële celwanden, ook glukosamine bevatten.

De sterke plaquevorming door *A. viscosus* (hoofdstuk IV) suggereert echter dat het extracellulair slijm een rol speelt bij dit proces. Ondanks het plaquevormende vermogen is de cariogeniteit van *A. viscosus* heel laag. Dit illustreert dat veel plaque niet noodzakelijkerwijs tot hoge cariesaktiviteit leidt.

Een opvallend resultaat levert de bestudering van de interaktie van *A. viscosus* met de SPF mikroflora. Terwijl beide komponenten afzonderlijk weinig cariogeen zijn, leidt hun gezamenlijke aktie tot een hoge cariogeniteit. De verklaring zou kunnen zijn dat de dikke plaque die wordt gevormd door *A. viscosus* onderdak biedt aan mikro-organismen die wel veel zuur produceren maar niet in staat zijn zich in voldoende mate op het tandoppervlak te handhaven.

Deze hypothese wordt verder uitgewerkt met behulp van het gnotobiotisch diermodel (hoofdstuk V). Het blijkt dat de samenwerking van de plaquevormer *A. viscosus* met de zuurvormer *Strep. sanguis* bij ratten zowel op een saccharose als op een glukose dieet, leidt tot minder caries dan wordt veroorzaakt door *Strep. sanguis* alleen. In hoeverre dit wijst op de onjuistheid van de hypothese is moeilijk uit te maken omdat daarnaast tal van andere wisselwerkingen denkbaar zijn tussen beide mikro-organismen.

De bestudering van de interaktie van *A. viscosus* met *Strep. mutans* leidt tot eenzelfde konklusie. Het blijkt dat *Strep. mutans* C67-1 de vestiging van *A. viscosus* in de plaque verhindert, waarschijnlijk door de produktie van een antibakteriële stof. Vanwege zijn instabiliteit kan het chemisch karakter van de antibakteriële komponent niet worden vastgesteld. Het resultaat vestigt echter de aandacht op de mogelijkheid dat antibakteriële stoffen een rol spelen in het ekosysteem van de tandplaque.

Behalve *A. viscosus* zijn nog andere slijmvormende mikro-organismen behorende tot de geslachten *Rothia*, *Neisseria* en *Bacterionema*, uit de tandplaque geïsoleerd (hoofdstuk VI). Ook deze mikro-organismen bevorderen de plaquevorming bij ratten. Bij dezelfde ratten is bovendien gelet op tandvleesontsteking. De mate waarin tandvleesontsteking wordt waargenomen hangt samen met de hoeveelheid plaque, een relatie die ook door Theilade c.s.

(1966) in klinische experimenten werd vastgesteld. Het is opmerkelijk dat dit zo ongeveer het enige vaststaande gegeven is bij de verklaring van het ontstaan van tandvleesontstekingen.
Omtrent de aard van de mikrobiële produkten en hun werking op het tandvlees is verder nauwelijks iets bekend.

Hoofdstuk VII beschrijft de eigenschappen van het enzym fruktosyltransferase (levaansaccharase) dat wordt gemaakt door *A. viscosus*. Dit enzym zet saccharose om tot fruktaan.
Het enzym van *A. viscosus* wordt vergeleken met de fruktosyltransferase van andere bakteriën.

Referenties

Black, G.V.: Dr. Black's conclusions reviewed again. Dent. Cosmos **40**: 440-451 (1898).

Guggenheim, B.: Extracellular polysaccharides and microbial plaque. Intern. dent. J. **20**: 657-678 (1970).

König, K.G., Guggenheim, B. and Mühlemann, H.R.: Modifications of the oral bacterial flora and their influence on dental caries in the rat II. Helv. odont. Acta **9**: 130-134 (1965).

Miller, W.D.: Die Mikroorganismen der Mundhöhle. Georg Thieme, Leipzig, 1889.

Theilade, E., Wright, H.W., Börglum Jensen, S. and Loë, H.: Experimental gingivitis in man. II. J. periodont. Res. **1**: 1-13 (1966).

Williams, J.L.: A contribution to the study of pathology of enamel. Dent. Cosmos **39**: 269-301 (1897).

Caries Res. 6: 203–210 (1972)

Methodological Aspects of Gnotobiotic Caries Experimentation

Preliminary Investigations into the Microbial Ecology of Dental Plaque

J. S. VAN DER HOEVEN, F. H. M. MIKX, A. J. M. PLASSCHAERT and K. G. KÖNIG

Institute of Preventive Dentistry, Medical Faculty, University of Nijmegen, Nijmegen

Abstract. A comparative study of caries activity is described on Osborne-Mendel rats reared germ-free and mono-associated with *Strep. mutans* OMZ 176 or E 49, or associated with one of these strains at the same time as the animals were conventionalized with an SPF microflora. The influence of the plaque matrix was studied with the aid of soft wood getting impacted in the fissures. Conventionalization with faeces from SPF rats resulted in a markedly lower caries activity compared with mono-infection. The presence of wood shavings in the diet and subsequent impaction in fissures did not result in increased caries activity when the flora consisted exclusively of microorganisms which formed sticky matrix polysaccharides.

Key Words
Caries, rats
Experimental caries
Gnotobiosis
Plaque matrix
Streptococcus mutans

After two decades of gnotobiotic research in the field of dental caries there are still some gaps in the knowledge necessary to explain the differences in response of conventional rats and animals harbouring a controlled microflora.

The interaction of a known strain of *Strep. mutans* with the conventional and antibiotic-depressed oral flora ('relative gnotobiosis') in rats has been investigated by KÖNIG *et al.* [1965]. Depression of the indigenous flora with erythromycin resulted in a marked decrease in the number of fissure lesions and an increase in lingual smooth surface lesions. However, no comparison has been made with respect to the cariogenic activity of streptococci between mono-associated rats and rats with a conventional microflora, both groups starting from the germ-free state. In order to study the influence of the indigenous flora on the cari-

ogenic activity of *Strep. mutans*, half of the rats of germ-free litters were mono-associated with *Strep. mutans* and the other half conventionalized with a mixture of *Strep. mutans* and the faecal flora of SPF rats.

Formation of extracellular polysaccharide by streptococci has been suggested to promote caries not only on smooth surfaces but also in fissures of rat molars [KONIG *et al.*, 1965]. However, the role of polysaccharide as a matrix for cariogenic microorganisms in fissures can also be fulfilled by impactions of spongy material like wood which increased the number of fissure lesions in rats when added to a high sucrose diet [KÖNIG, 1965]. It was suggested that fissures impacted with wood either retain more substrate, or harbour more acidogenic organisms, or are less accessible to the flow of neutralizing saliva and, therefore, more likely to develop caries. In the present study the interaction of wood impaction with the oral flora was investigated by comparing the caries activity of *Strep mutans* in mono-associated and conventionalized rats in screen bottom cages and in cages with wood shavings. The experimental design offered an opportunity to compare the caries activity of rats inside and outside of isolators

Materials and Methods

Animals and Treatments

Three litters of germ-free Osborne Mendel rats were transferred 12 days after birth to screen bottom cages without bedding in order to maintain the persistence of empty fissures The animals were fed γ-irradiated (2 5 Mrad) diet 2000 f until weaning. This diet was a modification of the diet 2000 [KEYES and JORDAN, 1964] in which the 56% sucrose component was replaced by wheat flour

At the weaning age of 26 days, three blocks of 8 litter-mates were formed, ear-marked and distributed at random among the cages in the two isolators Two cages in each isolator had screen bottoms without bedding, the other two had a stainless steel bottom with wood shavings From each isolator, two cages (one with and one without wood shavings) were removed to be conventionalized The rats remaining in the isolators were mono-associated with *Strep mutans* OMZ 176 and with *Strep mutans* E 49, respectively. The inoculum was applied by one single swabbing with a cotton stick The rats to be conventionalized were treated in the same way with a mixed inoculum of the respective test strain and in addition, faeces of SPF-Osborne-Mendel rats. During the 35-day experimental period the rats received γ-irradiated (2 5 Mrad) diet 2000 V [FITZGERALD, 1968] Food and water were available *ad libitum* The arrangement of the treatments is shown in table I.

Table I. Arrangement of treatments

	1	2	3	4	5	6	7	8
Number of rats	3	3	3	3	3	3	3	3
Bedding[1]	−	+	−	+	−	+	−	+
Strain inoculated								
OMZ 176	+	+	−	−	+	+	−	−
E 49	−	−	+	+	−	−	+	+
Conventional (SPF) flora[2]	−	−	−	−	+	+	+	+

[1] Bedding + : supplying wood shavings as bedding and in the diet.
[2] Conventionalized: inoculated with suspension of faeces from SPF-rats.

Microorganisms

Suspensions for the inoculation with *Strep. mutans* strain OMZ 176 [GUGGENHEIM, 1968] and strain E 49 [FITZGERALD, 1968; BRATTHALL, 1970] were prepared from 15-hour cultures in brain heart infusion (Difco) containing 2% glucose. 100 ml were centrifuged and the cells were suspended in 5 ml saline. The inoculum used for conventionalization was prepared just prior to the inoculation by homogenizing 1 g of fresh faeces of SPF-OM rats in 5 ml saline and mixing this with an equal volume of a suspension of the test strain.

Methods of Evaluation

After 3 and 10 days and at the end of the experiment mouth swabs and faeces from all treatments were examined. The presence of the inoculated strain and possible contaminations of the mono-contaminated isolators was checked by the following method. The tip of the mouth swab was transferred to a 5-ml screw-cap bottle containing a transport medium [DE STOPPELAAR *et al.*, 1969]. This sample was shaken on a Vortex mixer and suitable dilutions of this suspension in saline were spread on blood agar, sucrose agar [DE STOPPELAAR *et al.*, 1967] and Mitis Salivarius agar (Difco). The plates were incubated anaerobically in an atmosphere of 90% N_2 and 10% CO_2 for 4 days at 37 °C.

After sacrifice, the jaws were removed and fixed in 10% buffered formaline. Smooth surface caries was scored according to KEYES' method [1958]. The lower jaws were serially sectioned, stained and put on slides according to techniques described previously [KÖNIG *et al.*, 1958; KÖNIG, 1966; GREEN and HARTLES, 1967]. On the coded slides, the number of carious lesions in 16 fissures and the proximal areas of the right and left mandibular molars were counted under incident light at 16-times magnification. After decoding and tabulation, the data were subjected to analyses of variance.

Results

Microorganisms

Three and 10 days after inoculation, and at the end of the experi-

Table II. Weights of rats at the end of the experiment. Average and standard deviation for the separate factors sex, wood impaction and conventionalization

	Average weight, g	Standard deviation, g	Number of rats
♂	212.00	28.35	16
♀	177 37	23 47	8
Without wood particles (♀ N=4; ♂ N=8)	204.66	35.77	12
Wood particles (♀ N=4; ♂ N=8)	196 25	19.42	12
Mono-associated (♀ N=4; ♂ N=8)	208.08	34.47	12
Conventionalized (♀ N=4; ♂ N=8)	192.83	19.49	12

ment, the inoculated test strains could be recovered from rats on all treatments. In the mono-associated rats no contamination was found.

Weight Gains

Growth of the animals was normal on all treatments as judged by terminal average weight (table II). There was a normal sex difference in average weights.

Caries Incidence

The average numbers of carious fissures, carious smooth surface units and proximal lesions found at the end of the experimental period are presented in table IIIa and b. Strain E 49 induced a significantly higher caries activity than strain OMZ 176 in fissures and smooth surfaces (table IV, comparison C_1, $P_F<0.01$ and $P_F<0.05$, respectively).

The difference in caries activity was consistent for the three types of carious lesions (table IIIa). The conventionalization with faeces from SPF-rats resulted in a markedly lower caries activity compared to mono-association. This is shown by the mean values for fissure lesions, smooth surface lesions and proximal ones (table IIIa, b) and by the analysis of variance (table IV, C_2, $P_F<0.001$). The lower caries activity in the conventional state was observed in both groups of rats inoculated with OMZ 176 and E 49 (table IV, C_3).

Table IIIa Caries scores resulting from grouping the rats so as to reveal the mean influences of the 6 different experimental factors

	B	E	Prox
Strain OMZ 176	8 3	4 9	0 2
Strain E 49	11 0	8 6	4 5
Wood particles	10 0	5 3	2 3
Without wood particles	9 3	8 2	2 3
Mono-associated	14 3	9 6	4 0
Conventionalized	5 0	3 9	0 7

Average numbers per rat of all dentinal lesions (B), of buccal carious smooth surface areas (E) and proximal lesions (Prox), 12 rats per group

Table IIIb Fissure caries scores (B in table IIIa) resulting from subdivision of the rats according to the 8 treatments

	Strain	
	OMZ176	E49
Mono-associated		
Wood	11 7	16.0
No wood	13 6	16 0
Conventionalized		
Wood	5 0	7 3
No wood	3 0	4 7

Average numbers per rat of all dentinal lesions, B. N = 3 rats per group

The presence of wood shavings resulted only in small and insignificant increases in fissure lesions (table IIIb) and an insignificant decrease in the number of smooth surface lesions (table IIIa, IV, C_4). In table IV, C_5 shows that the wood impaction did not play a major role in both mono-associated and conventionalized rats.

Discussion

One objective of the present investigation was to determine a possible effect of impaction of a sort of matrix in the development of caries

Table IV. Analysis of variance of dentinal fissure lesions (B) and of buccal smooth surface lesions encountered in the 8 groups of 3 rats

	Source of variation				
	d.f.	fissure lesions		smooth surface lesions	
		SS	MS	SS	MS
Treatments	5	587.30	117.46[3]	402.32	80.46[2]
C_1 strain OMZ176 vs. E49	1		42.66[2]		80.67[1]
C_2 mono-associated vs. conventionalized	1		522.66[3]		192.66[2]
C_3 interaction $C_1 \times C_2$	1		2.66		48.17
C_4 wood vs. no wood	1		2.66		48.16
C_5 interaction $C_2 \times C_4$	1		16.66		32.66
Litters	2	58.34	29.17[1]	32.24	16.12
Remainder (error)	16	83.70	5.23	269.94	16.87
Total	23	729.34	–	704.50	–

d.f. = Degrees of freedom; SS = sum of squares; MS = mean square.
[1] $P_F < 0.05$.
[2] $P_F < 0.01$.
[3] $P_F < 0.001$.

in gnotobiotic rats. The impaction of wood particles from the diet and the bedding was found not to be an important variable in causation of fissure and approximal caries under the conditions of the present experiment. This seems not to be in accordance with the previous observation of KÖNIG [1965], that fissures impacted with wood developed more caries. However, in the present experiment the rats had been infected with strains of *Strep. mutans* which are known to form sticky extracellular polysaccharide. In the presence of a flora with a preponderance of microorganisms which cannot produce an own matrix as easily as *Strep. mutans*, the presence of an artificial matrix as in the previous experiment [KÖNIG, 1965] may be assumed to be more essential in the development of carious lesions than when polysaccharide formers are preponderant or the only microorganisms present. The trend towands higher caries rates on wood when OMZ 176 and E 49 were in competition with the SPF flora confirms this hypothesis.

In general, a significantly lower caries incidence was found in the conventionalized rats which harboured *Strep mutans* among other organisms, than in rats which were mono-associated with *Strep mutans* In a previous experiment the cariogenicity of the conventional SPF flora used in this laboratory was found to be very low [unpublished results] Therefore, the results obtained in the present experiment may be interpreted as a reflection of the decreased proportion of *Strep mutans* in the plaque of the conventionalized animals

It is not possible to draw further conclusions on the nature of the interaction of *Strep mutans* with the complex conventional flora on the basis of caries activity only The interactions of bacteria in plaque need to be studied in future experiments on gnotobiotic rats and *in vitro,* using a limited number of strains in carefully selected combinations

Acknowledgements

The authors wish to thank Dr VAN KOOY, Institute for Atomic Sciences in Agriculture (ITAL), Wageningen, for sterilizing the test diets, Mr LUKASSEN and Mr KOEDAM, Central Animal Laboratory, Medical Faculty, University of Nijmegen, for taking care of the test animals

References

BRATTHALL, D Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans* Odont Rev *21* 143–152 (1970)

FITZGERALD, R J Dental caries research in gnotobiotic animals Caries Res *2* 139–146 (1968)

GREEN, R M and HARTLES, R L A modification to an established technique for the assessment of fissure caries in the rat Arch oral Biol *12* 427–428 (1967)

GUGGENHEIM, B Streptococci of dental plaques Caries Res *2* 147–163 (1968)

KEYES, P H Dental caries in the molar teeth of rats II A method for diagnosing and scoring several types of lesions simultaneously J dent Res *37* 1088 (1958)

KEYES, P H and JORDAN, H V Periodontal lesions in the Syric hamster III Arch oral Biol *9* 377 400 (1964)

KONIG, K G Caries resistance in experimental animals, in WOLSTENHOLME and O'CONNER Ciba Foundation Symposium on caries-resistant teeth, pp 87–106 (Churchill, London 1965)

KONIG, K G Moglichkeiten der Kariesprophylaxe beim Menschen und ihre Untersuchung im kurzfristigen Rattenexperiment, p 67, p 77 (Huber, Bern 1966)

Caries Res 8 193–210 (1974)

III

A Slime-Producing Microorganism in Dental Plaque of Rats, Selected by Glucose Feeding

Chemical Composition of Extracellular Slime Elaborated by *Actinomyces viscosus*, Strain Nyl

J. S. VAN DER HOEVEN

Institute of Preventive Dentistry, University of Nijmegen, Nijmegen

Abstract The administration of a glucose-rich diet to a group of O-M rats was followed by development of massive amounts of plaque. The predominating microorganism isolated from these plaques was a filamentous form identified as *Actinomyces viscosus A viscosus*, growing in dialyzed actinomyces broth (BBL) containing excess of glucose, produced a viscous culture from which extracellular slime was isolated by precipitation with ethanol

Fractionation of the extracellular slime on DEAE-Sephadex revealed the presence of two major components, one of which contained mainly glucose, while the other was more complex and contained glucosamine, hexose and protein

Antibodies could be produced against extracellular antigens which were found to be present in the slime but only accounted for a minor portion of the material

Key Words
Actinomyces viscosus
Glucose diet
Plaque
Polysaccharide, extracellular
Rat caries

The influence of environmental factors on the microbial composition of dental plaque is clearly illustrated by the role of dietary sucrose.

Frequent consumption of sucrose favours the establishment of streptococci synthesizing extracellular glucan in plaque [CARLSSON, 1967; DE STOPPELAAR, 1970]. The presence of glucan as a constituent of plaque matrix has been demonstrated by biochemical analyses and electronmicroscopic investigations. Glucan synthesis is believed to play an important role in the accumulation of plaque. The ability to elaborate capsular material or extracellular slime in the absence of sucrose is fairly widespread among plaque microorganisms: *M. mucilaginosus* [BOWDEN, 1969; KOCUR *et al*, 1971], lactobacilli [HAMMOND, 1967], rothia [RITZ, 1963],

neisseria [PARKER and CREAMER, 1971] and streptococci [GUGGENHEIM, 1970b]. However, it is not known which conditions favour the growth of these particular species in plaque and whether the production of the extracellular polymers is essential in plaque formation. The aim of the present investigation was to follow the microbial composition of plaque especially in relation to slime-producing microorganisms, when sucrose was completely absent in the diet and was replaced by glucose. It was expected that particular types of microorganisms synthesizing substantial amounts of extracellular material of high molecular weight from glucose would appear. These experiments were carried out in the rat and it was observed that *Actinomyces viscosus* accumulated in the plaque of glucose-fed animals. *A. viscosus* was originally isolated from hamsters and its metabolism has been studied by HOWELL and JORDAN [1963]. Further investigations on the metabolic capabilities of one of the strains establishing spontaneously on a glucose diet are described here.

Materials and Methods

Enrichment and Isolation of Microorganisms

Two dietary groups of 16 young conventional Osborne-Mendel rats were formed using two animals from each of 8 litters. The animals of each group were distributed at random among 8 cages, and fed on a glucose or a sucrose diet (56% glucose, or sucrose, 28% skim milk; 8% wheat flour, 7% yeast extract, 1% NaCl), and tap water *ad libitum* From each group, two animals from the same cage were sacrificed by decapitation at 2-week intervals. The plaque was removed from the smooth surfaces of the upper molars and placed into transport medium [DE STOPPELAAR, 1969] The plaque was homogenised by ultra-sonication (Branson sonifier provided with microtip) 3 times for 10 sec, with 30 W output, and 10-fold serial dilutions were spread on blood agar, brain-heart-glucose (1%) agar and trypticase-yeast extract (T-Y) sucrose agar [DE STOPPELAAR *et al*, 1967]. The agar media were incubated both aerobically, and anaerobically in an atmosphere of 90% N_2, 10% CO_2, for 5 days at 37 °C Colonies were inspected under a dissecting microscope and each morphological type was examined in wet-indian ink preparations for the presence of extracellular polymer. Positive samples were subcultured for further investigation

Identification of Microorganisms

Biochemical tests. Tests for catalase, indole, nitrate reduction, methyl red reaction, acetoin, starch hydrolysis and gelatin hydrolysis were performed according to the procedures recommended by the 'Subgroup on Taxonomy of the Microaerophilic actinomycetes'. Carbohydrate fermentation tests were done in a basal medium, supplemented with 1% carbohydrate Incubation was for 14 days in an atmosphere of 90% N_2 and 10% CO_2.

Cell wall composition and serology of one of the isolated strains, *A viscosus* strain Ny1, were studied by Mr G H BOWDEN [(The London Hospital Medical College) Preparation and analyses of cell walls have been described elsewhere [BOWDEN and HARDIE, 1973] Trypsin-treated cell walls and an acid extract of cells of Ny1 were provided by G H BOWDEN

Chemical Analyses

Carbohydrate was determined with the phenol-sulfuric acid method [DUBOIS *et al*, 1956] Protein was measured according to LOWRY *et al* [1951] Aminosugars were assayed according to CESSI and PILIEGO [1960] Glucose was determined with glucose oxidase-peroxidase and *o*-dianisidine Fructose was measured by the sulfuric acid-cysteine reaction of DISCHE [1962]

Glycerol was assayed with glycerokinase after hydrolysis for 1 h in 3 N HCl at 100 °C, and pyruvate with lactate dehydrogenase after hydrolysis for 1 h in 1 N HCl at 100 °C Uronic acids were estimated with the carbazole method [DISCHE, 1947] Phosphate was determined according to CHEN *et al* [1956] Sulfate was determined turbidimetrically as $BaSO_4$ [BERGLUND and SORBO, 1960] DNA and RNA were assayed with the method of SCHNEIDER [1946] Lactic acid was determined with (D+L)-lactate dehydrogenase (Boehringer)

Hydrolysis conditions The optimal times of hydrolysis for the release of glucose and hexosamine were determined for crude slime and these values were used for all other samples Hydrolysis for 2 h in 1 N HCl at 100 °C in evacuated tubes gave optimal results for hexosamine The highest yield of glucose was obtained after hydrolysis for 2 h in 2 N H_2SO_4 HCl hydrolyzates were dried in a vacuum desiccator over NaOH and the H_2SO_4 hydrolyzate was diluted to 0 4 N and subsequently applied to a column of Dowex 1 (H^+) and separated in a neutral sugar and an aminosugar fraction, as described by SPIRO [1966]

Paper chromatography Paper chromatography of neutral and aminosugar fractions was performed on Whatman 1 MM paper using butanol/pyridine/water, 6 4 3 [JEANES *et al*, 1951] Reducing sugars were detected with aniline phthalate and aminosugars with ninhydrin

Gas chromatography Neutral sugars were identified as trimethyl-silyl-derivatives To water-free hydrolyzate 1 ml Tri-Sil (Pierce Chemical Company) was added and the mixture was then heated for 1 h at 80 °C in a screw-cap vial Pyridine was removed in a rotating evaporator at 30 °C and replaced by benzene Gas chromatography was performed under the conditions described by GUGGENHEIM [1970a] Saturated fatty acids C2-C5 were assayed semi quantitatively by gas chromatography The millipore filtered fermentation broth was made 10% w/v formic acid and then injected onto the column of a Perkin Elmer F_{11} chromatograph A glass column (3 mm × 1 5 m) packed with Porapak Q S (Waters Ass, Cheshire) was used [HENKEL, 1971] Samples of 1 μl were chromatographed isothermically at 160 or 190 °C, other conditions were carrier gas nitrogen, 25 ml/min flame ionisation detector 230 °C and injection port 210 °C Ethanol could also be assayed with this method

The acetate content of extracellular slime was gas-chromatographically determined in samples which were hydrolyzed in 1 N HCl at 100 °C for 1 h

Ion-exchange chromatography. Aminosugars and muramic acid were identified and assayed using an amino acid autoanalyzer.

Serological Methods

Rabbits were intravenously immunized 4 times at 3-week intervals. One week after the last injection, a test bleed was made. The first dose was 0.2 ml of a 1-percent solution of extracellular slime in 0.85% saline and the following doses were 0.5 ml of the same solution each. The cells used for immunization were grown in dialyzed actinomyces broth in screw cap vials. The cells were centrifuged and washed twice with 0.85% saline and suspended in saline.

Serological activities were tested by the double diffusion (Ouchterlony).

Growth of A. viscosus *Ny1 and Production of Extracellular Slime*

Strain Ny1 was grown in batch (48 h, stationary culture) or continuous culture in a 500-ml fermentor, at a dilution rate of 0.1 in each of the following media: dialyzed actinomyces broth (BBL) containing 25 or 175 mM glucose; dialyzed brain-heart infusion (Difco) broth containing 200 mM glucose; casitone 0.2% (w/v) medium containing 5 or 50 mM glucose and casitone 1.2% (w/v) containing 180 mM glucose. The casitone media were prepared from a basis medium as described by CARLSSON [1970, table VI] supplemented with casitone (Difco) and glucose as indicated above.

All cultures were incubated at 37 °C in an atmosphere of 90% N_2/10% CO_2, and pH was kept constant at 7.0. Balanced growth in continuous culture was maintained as judged from the fact that viable cell counts and A550 nm determined at regular intervals remained constant for at least two generations. Five-millilitre samples were taken for determination of the products of anaerobic fermentation of glucose. The production of slime was followed by measurement of the viscosity of the culture supernatant fluid (centrifugation 30 min, 30,000 *g*) in an Ostwald viscosimeter. The amount of crude polymer in each culture was determined by the addition of 2 volumes of absolute ethanol to the culture supernatant. The precipitate was collected by filtration on a Buchner filter, extensively washed with 70% ethanol and finally with absolute ethanol, and then dried under vacuum.

Isolation of Crude Extracellular Slime

When grown in dialyzed actinomyces broth containing glucose (175 mM/ml), strain Ny1 produced a very viscous culture from which cells could be centrifuged only with difficulty. Slime was precipitated from the centrifuged (30 min at 30,000 *g*) culture by the addition of 2 volumes of ethanol with stirring. No further precipitation of non-dialyzable material occurred on further addition of ethanol to the culture supernatant fluid. Any extracellular material adhering to cells was discarded and stored for later investigation. The precipitate was collected by filtration or centrifugation, and dispersed in saline by ultrasonic dispersion. Non-sediment cells and some protein were removed from solution by adjustment of the pH to 3 with 4 N sulfuric acid. The precipitate was removed by centrifugation. Polymers were then reprecipitated with ethanol and solubilized again; this step was repeated once. The polymer solution was then dialyzed against distilled water and freeze-dried. All separation steps were carried out at 4 °C.

Fractionation of Extracellular Slime

Samples (250 mg) of the lyophilized slime were fractionated by ion-exchange chromatography on DEAE-Sephardex A-50 The column (2 5×40 cm) was eluted with a gradient from 0 to 1 M NaCl in 0 05 M tris-HCl, pH 8.0, at a flow rate of 8 ml/cm²/h. Peaks were pooled as indicated (fig. 1), dialyzed and freeze-dried (fractions 1 and 2) and then subjected to gel chromatography on Sephadex G-100 Samples were eluted (column 1.6×30 cm) with 1% NaCl Fractions (5 ml) of the eluates were measured for absorbance at 260 nm, and 0.2-ml samples of each second fraction were analysed for carbohydrate and aminosugar Chloride concentration in the gradient was measured with a solid-state chloride electrode (Orion). Alternate fractions were tested for serological activity

In two experiments, all serologically active fractions eluted from DEAE-Sephadex were treated as described above and then, after purification on Sephadex G-100, subjected to isoelectric focusing Isoelectric focusing was carried out with the Uniphor apparatus (LKB) using 1% ampholine, pH 3–5 (LKB), in a density gradient of 0–50% sucrose or glycerol. A 220-ml column was used and the material was focused for 72 h The terminal voltage was 500–550 V and the current was constant at 0.4–0 5 mA Fractions (5 ml) were eluted from the column and, after determination of pH, each fraction was dialyzed against distilled water The carbohydrate, aminosugar content and the absorbance at 260 nm of each fraction were then determined and tests for serological activity were carried out

Results

Microorganisms Selected by Glucose Feeding

Heavy plaque development was observed in animals of the glucose and the sucrose groups sacrificed after 4 weeks.

In the sucrose group, considerable numbers (mean 40%, range 30–65%, expressed as counts on T-Y sucrose agar relative to total counts on anaerobic blood agar) of extracellular polysaccharide-producing streptococci were consistently found in plaque. Polysaccharide-producing streptococci were identified according to their morphological appearance on T-Y sucrose agar [De Stoppelaar *et al.*, 1967].

In the samples of the glucose group, high numbers of one particular type of colony were found. The mean percentage of this type of colony was 60% (range 35–85%) of total counts on anaerobic blood agar. West-indian ink preparations revealed that loose extracellular slime was produced by this microorganism on blood and glucose agar. The microorganism, which was found in all animals of the glucose group, was only incidentally observed in the original plaque flora and in the sucrose-fed animals The extracellular slime-producing microorganism, which was predominant in the glucose group, was further investigated.

Table I Biochemical reactions of rat strains of *A viscosus* (including strain Ny1)

Test	N	Test	N
Glucose	6	Catalase	6
Galactose	6	Indole	0
Arabinose	0	Nitrate	6
Cellobiose	0	Acetoin	0
Lactose	6	Starch hydrolysis	4
Sucrose	6	Esculin hydrolysis	0
Raffinose	6	Gelatin hydrolysis	0
Inositol	6	Methyl red	6
Xylose	3		

Number of positive strains of 6 strains tested

Positive in fermentation test means pH <5.5 In the absence of added carbohydrate, the basal culture medium reaches a pH of 6.5

Identification of the Slime-Producing Microorganism

Biochemical characteristics of 6 isolates from different animals are given in table I.

Morphology. The colonial and cellular morphology of the 6 isolates were similar to those of the type species of *A. viscosus* strain ATCC 15987. In particular, the adhesiveness of aerobically grown colonies to the agar medium was typical. One of the isolates, strain Ny1, was selected for further studies.

When Ny1 was grown at low growth rate in a casitone medium containing 5 mM glucose, coccoid populations were observed. At higher growth rates, the coccoid cells transformed to branching filamentous cells with club-like extremities which represented the common morphology in all liquid cultures. This result suggested that morphology is related to specific growth rate.

Cell wall composition of Ny1. Amino acids[1] in cell-wall mucopeptide: ornithine, lysine, glycine, glutamic acid, alanine, valine and leucine were present. No di-amino pimelic acid was found. Reducing sugars[1]: rhamnose, glucose (trace), galactose (trace) and a deoxyhexose almost certainly 6-deoxytalose [HAMMOND *et al.*, 1973] were found. Aminosugars: glu-

[1] Determination of amino acids and reducing sugars in trypsin-treated cell walls was performed by G. H BOWDEN

Table II. Growth of *Actinomyces viscosus* and production of extracellular slime

Medium	Glucose, mM	Batch cultures			Continuous cultures (D = 0.1)		
		A550 nm	ηrel	EtOh prec	A550 nm	ηrel	EtOH prec, g/l
Casitone 0.2%	50	–	1.1		3 1	1.1	–
Casitone 1.2%	180	4.4	1.1	0.1	14.9	1.3	0.1–0.2
Brain-heart infusion (dialyzed)	200	4.1	1.4		14.5	1.8	n.d.
Actinomyces broth (dialyzed)	25	2.8	1.2		11.2	1,3	n.d.
Actinomyces broth (dialyzed)	175	4.7	1.7	0.3	15.5	2.9[1]	2.0–4.8

All experiments were performed at constant pH 7.0 under an atmosphere of 90% N_2/10% CO_2.

ηrel = η culture supernatant/η medium; EtOH prec = dry weight (g/l) of material precipitated by the addition of 2 volumes of ethanol to the culture supernatant fluid.

[1] In some experiments, values up to 8.0 have been observed.

cosamine and muramic acid were found to be present in the ratio 1:1. A trace of galactosamine might be present.

Serological findings. The acid extract of Ny1 gave a positive precipitation reaction with antisera against ATCC 15987. Lines of identity were observed between acid extracts of Ny1 and ATCC 15987 with antisera against each of both strains. It was concluded that Ny1 was a strain of *A. viscosus* serotype 1, which is the rodent serotype [GERENCSER and SLACK, 1969].

Appearance of Extracellular Slime from A. viscosus *Ny1*

Some experiments were performed in order to determine the effect of the growth medium on the production of extracellular slime. The results are summarised in table II.

In dialyzed actinomyces broth, significantly more slime was produced when excess of glucose was present. A relatively low amount of slime was produced in brain-heart infusion plus 250 mM glucose and virtually no

Table III. Anaerobic fermentation of glucose by *A. viscosus* Ny1 in continuous cultures (D = 0.1, pH 7.0; atmosphere 90% N_2/10% CO_2)

Medium	Glucose mM	Glucose utilized mM	A550 nm	Dry weight of cells mg/100 ml	L-lactate mM	acetate mM	ethanol mM
Casitone 0.2%	5	4.96	1.5	32	0.1	30.2	0.0
Casitone 0.2%	50	21.9	2.1	55	4.0	31.0	0.2
Casitone 1.2%	180	160.0	14.9	n.d.	166.5	22.5	0.8
Actinomyces broth (dialyzed)	25	24.3	11.2	210	33.8	11.4	n.d.
Actinomyces broth (dialyzed)	175	168.2	15.5	290	177.0	13.0	5.9

slime in casitone plus 180 mM glucose in spite of the fact that these latter two media supported excellent growth. Since slime was mainly constituted of carbohydrates, glucose utilization in some of the media used was also considered (table III).

Glucose utilization was found to be rather similar in both rich media: casiton (1.2% casitone, 180 mM glucose) and actinomyces broth plus (175 mM glucose).

Assuming that the fermentation of 1 mol glucose maximally yielded 2 mol lactate or acetate, it appeared that in actinomyces broth plus 25 mM glucose, the amounts of lactate and acetate accounted for almost all the available glucose, whereas in actinomyces broth plus 175 mM glucose approximately 60% of all utilized glucose was converted to lactate and acetate.

Chemical Composition of Extracellular Slime

Paper chromatography of hydrochloric and sulphuric acid hydrolysates of extracellular slime revealed two predominant sugar components, one with the same mobility and colour as glucosamine on ninhydrin-treated chromatograms and the other with the same characteristics as glucose on aniline hydrogen-phtalate-treated chromatograms. Table IV shows the results of several chemical analyses on extracellular slime. A discrepancy was observed between the results of the determination of glucose and carbohydrate in extracellular slime in that the glucose accounted for only 65% of the carbohydrate, while no other sugars could be detected by paper chromatography.

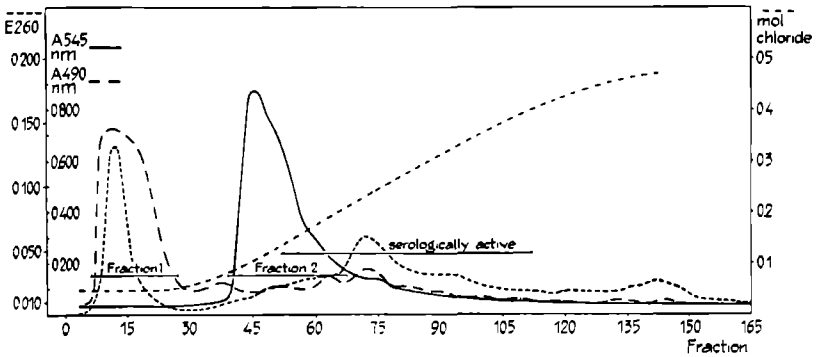


Fig. 1. Fractionation of extracellular slime of Ny1 on DEAE-Sephadex A-50. Elution with sodium chloride in 0.05 mol Tris-HCl, pH 8.0. Fractions 1, 2, and serologically active material were obtained as indicated. In all figures: — = A545 nm, aminosugar (Cessi).

The results of paper chromatography were confirmed by identification of aminosugars with the amino acid analyzer. Except glucosamine (50.2%), only a very small amount of galactosamine (0.8%) and a trace of muramic acid were found. Acetyl groups were present in fairly high amount and were derived exclusively from *N*-acetyl bonds since no *O*-acetyl was detected with hydroxylamine [HESTRIN, 1949]. A small amount of phosphate, 0.2–0.3%, was found in all batches of extracellular slime.

The following components were found to be absent: fructose, glycerol, pyruvate, sulfate, uronic acids, DNA and RNA.

Fractionation of Extracellular Slime

The elution pattern of extracellular slime from DEAE-Sephadex A-50 in a representative experiment is shown in figure 1.

Material rich in carbohydrate (fraction 1) passed straight through the column. A peak containing hexosamine eluted at higher ionic strength (fraction 2). Fractions 1 and 2 from DEAE-Sephadex were dialyzed against water, freeze-dried and weighed (table IV). Recovery from DEAE-Sephadex was approximately 90%. Separate fractions of the gradient were tested for serological activity against unfractionated slime antiserum in double diffusion. Serologically active material was found to elute from approximately 0.15 to 0.4 M sodium chloride.

The serologically active fractions from DEAE-Sephadex accounted for 10–15% of the material that was applied onto the column.

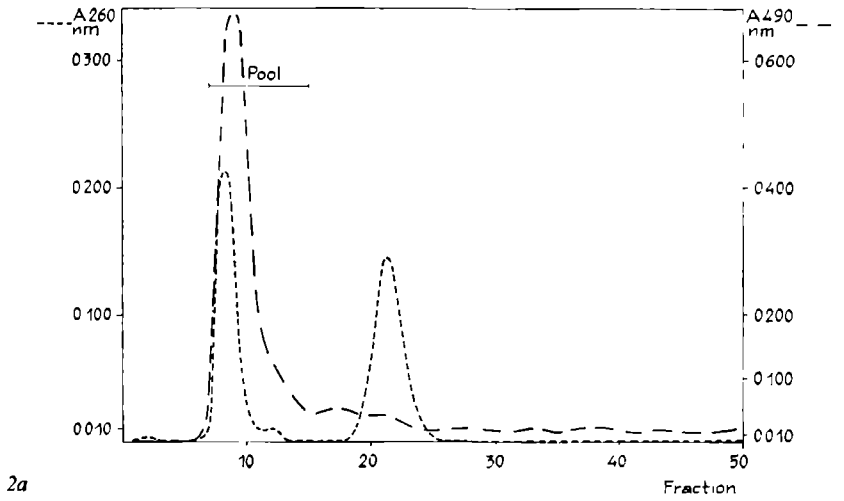


Fig. 2. Gel chromatography on Sephadex G-100 of fraction 1 (a), fraction 2 (b) and serologically active material (c) of extracellular slime obtained from DEAE-Sephadex. Fractions were pooled as indicated.

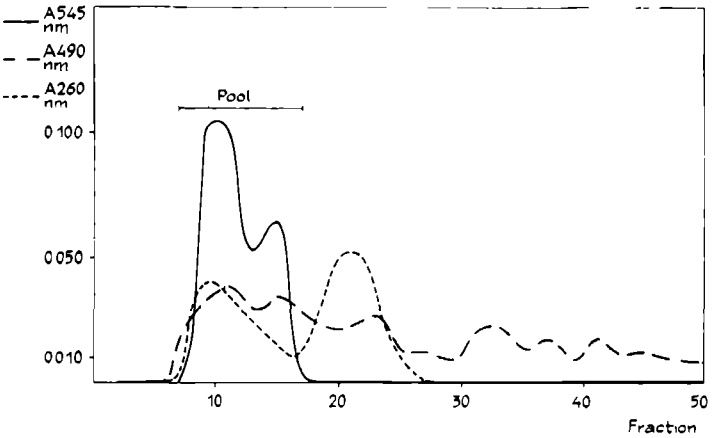
Fractions 1, 2 and serologically active material from DEAE-Sephadex were further purified by gel filtration on Sephadex G-100 (fig. 2a–c). Most carbohydrate-containing material of fraction 1 and serologically active material appeared in the void volume of the column (fig. 2a, c). In eluates from Sephadex G-100, a peak of UV-absorbing material was present at fraction 21 (fig. 2a–c).

The results of isoelectric focusing of serologically active material are shown in figure 3. It was observed that serologically active material was focused at remarkably low pH. Two pools of serologically fractions were constituted in a way that pool a contained the aminosugar peak (fractions 22–34, Ip approx. 3.6) and pool b contained the remaining active fractions (36–38, Ip approx. 2.5).

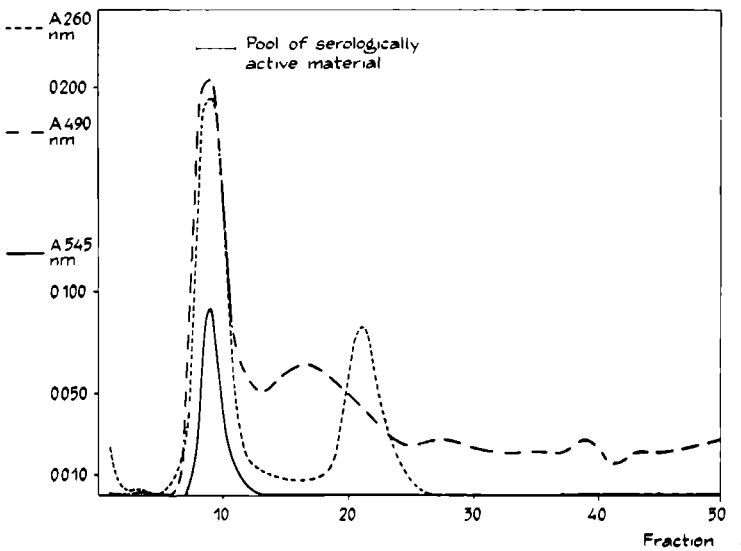
Chemical Composition of Fractions

The results of several chemical analyses on fractions 1 and 2 are shown in table IV.

Fractions 1 was mainly composed carbohydrate (86.5%). No sugars other than glucose were found by gas-chromatographic analysis. A small amount of aminosugar was found with the Cessi method but this might be



2b



2c

due to some interfering reaction since no aminosugars were found by analysis on the amino acid analyzer.

Fraction 2 contained aminosugar, protein and some carbohydrate. Only glucosamine was found to be present in the aminosugar fraction. A detailed analysis of the neutral sugar fraction has not been made, but, except glucose, other peaks were observed.

Table IV Chemical composition of extracellular slime produced by *Actinomyces viscosus* Ny1

	Protein %	Carbohydrate %	Glucose %	Glucose μmol/10 mg	Aminosugar %	Glucosamine %	Glucosamine μmol/10 mg	Galactosamine %	Acetyl μmol/10 mg	Material recovered from DEAE-Sephadex, %
Extracellular slime	12 0	15 2	10 0	5 6	57 5	50 2	27 9	0 8	17 4	
Fraction 1	0 1	86 5	74 2	41 2	0 7	0	0 0	0 0	1 0	10–20
Fraction 2	9 1	5 6	1 4	0 8	65 5	60 1	33 4	0 0	7 4	70–90
IEF pool a	5 3	25 3	+	+	42 0		+	+	+	
IEF pool b	8 1	64 0	+	+	3 3		+	+	+	

Extracellular slime was prepared from the culture supernatant by ethanol precipitation Fractions 1 and 2 were obtained by elution from DEAE-Sephadex A-50 and subsequent gel filtration on Sephadex G-100 Aminosugar was assayed according to CESSI and PILIEGO [1960] and glucosamine, galactosamine were determined with an amino acid autoanalyzer Data in the table represent means of triplicate measurements on two different batches which were generally very close

+ = not determined

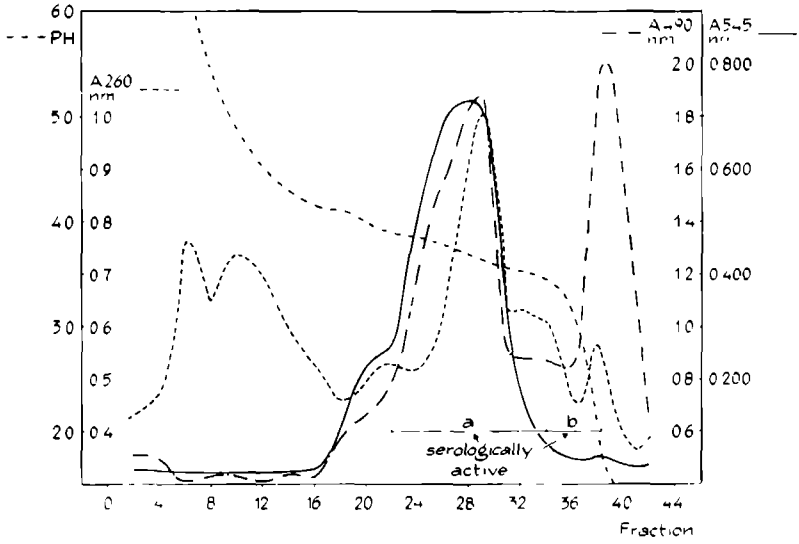


Fig. 3. Isoelectric focusing of serologically active components in extracellular slime of Ny1. Material was prepared by ion-exchange chromatography and subsequent gel chromatography and focused in a 0- to 50-percent sucrose gradient containing 1% ampholyte. Pools a and b were constituted as indicated.

Chemical Composition of IEF Pools

The results of some analyses on pools a and b are shown in table IV. Neutral sugars in pool b accounted for 64% of the material, and the ratio of the neutral sugars found to be present was about glucose/mannose/galactose = 2:1:1.

Serological Results

It was found that antibodies could be induced against components present in extracellular slime of Ny1. Antisera to whole cells or extracellular slime were shown to react identically, although the former generally gave stronger reactions. All tests recorded were, therefore, carried out with whole-cell antisera. A suitable whole-cell serum and also an antiserum prepared against cell walls was kindly provided by Prof. GUGGENHEIM (Zürich).

Extracellular slime showed at least 3 lines in double diffusion, 2 of which were identical to lines of the acid extract. IEF pool a showed 3 lines which indicated that the extracellular antigens could not be easily

separated by isoelectric focusing. IEF pool b showed a single line. This material reacted strongly with cell wall antiserum showing again a single line.

Discussion

Substrate Effects and Microbial Interactions

Profound changes in the oral flora and concomitant development of massive amounts of plaque followed the administration of high glucose or sucrose diets. The strong increase of the number of streptococci synthesizing extracellular polysaccharide in the sucrose group underlined the well-known favourable effect of sucrose on their establishment and proliferation [KRASSE 1966; KRASSE and CARLSSON, 1970]. The observed predominance of *A. viscosus* in the glucose group illustrated the importance of the diet as a factor that determines the composition of the microflora in the plaque.

A. viscosus was originally isolated from hamsters kept on a high sucrose diet [KEYES *et al.*, 1963]. In the present experiment, it was observed that sucrose favoured the establishment of streptococci rather than of *A. viscosus*. In subsequent tests [MIKX *et al.*, 1973] however, *A. viscosus* was found to maintain in very high numbers when inoculated in rats on sucrose diet, together with *Strep. mutans* and *Strep. sanguis*. This might indicate that streptococci can prevent the proliferation of low numbers of *A. viscosus* in developing plaque, but not when large numbers of this microorganism are supplied by inoculation. The high plaque-forming ability of *A. viscosus* [VAN DER HOEVEN *et al.*, 1973] was attributed to the production of extracellular slime. The presence of slime in broth cultures of *A. viscosus* was earlier noted by JORDAN and KEYES [1964], but its characteristics have not previously been described.

Factors Influencing the Synthesis of Extracellular Slime

The ability to produce extracellular material of high molecular weight in glucose broth must be distinguished from sucrose-dependent levan synthesis by *A. viscosus* [HOWELL and JORDAN, 1967]. The conditions involved in the production of slime are complicated in that excess of glucose and other chemical factors as well as cultural conditions influence the output. The fact that the highest yield of slime per litre culture was obtained in continuous cultures suggested that the slime was produced and released in the logarithmic phase of growth. It was observed that ex-

cess of glucose markedly stimulated slime production in actinomyces broth, but that virtually no slime was produced in casitone medium containing the same amount of glucose.

The nature of the stimulatory factors is unknown. That yeast extract was not responsible, is suggested by the observations in a pilot experiment: addition of 1% yeast extract to the casitone medium did not stimulate slime production, whereas the addition of 25 vol% of actinomyces broth did. The striking difference which was found for slime production on actinomyces broth and casitone is not observed for the other metabolic performances studied. At an equal growth rate and approximately the same density of cells, glucose utilization and amounts of fermentation products were found to be rather similar on both media. However, it must be noted that not all fermentation products (e.g. formic and succinic acids) were considered and the possible synthesis of intracellular polysaccharide was not determined.

An interesting finding was the low production of lactate by *A. viscosus* in media containing relatively little protein (casitone 0.2% media, table III). This characteristic might explain the low cariogenicity of the microorganism when tested in gnotobiotic rats [VAN DER HOEVEN *et al*, 1973].

The amount of glucose could not account for the high quantity of acetate produced in the glucose-limited (5 mM) culture (table III), thus it must be concluded that acetate was also derived from amino acid breakdown. No attempt will be made to discuss the results of table II with regard to the fermentation balance of *A. viscosus* as proposed by PINE [1970].

Chemical Composition of Extracellular Slime

The extracellular slime contained two major components with distinctly different chemical composition. Fraction 1 consisted predominantly of glucose, no other neutral sugars or aminosugars could be detected. This material might be related to intracellular polysaccharide and its presence might be attributed to leakage because of disrupted permeability barriers. In fraction 2, glucosamine protein, some carbohydrate and *N*-acetyl were found. Except glucose, some other sugars not yet identified were present in this fraction. The fact that these sugar components were not detected by paper chromatography is not surprising, since resolution in the chromatographic system used was not found to be optimal, and they were present in small amounts. The observation that fraction 2 is slightly retarded on DEAE-Sephadex despite the virtual lack of negatively charged

groups cannot be easily explained but may result from hydrophobic interactions between the polysaccharide chain and the dextran matrix. The molar ratio of glucosamine to *N*-acetyl in slime was found to be lower than 1. However, it was not tested whether complete hydrolysis of acetate was achieved after 1 h in 1 N HCl and it might well be that all glucosamine is acetylated in the original material.

The virtual absence of muramic acid in extracellular slime demonstrated that the preparations were free from cell wall mucopeptide. It was found that trypsin-treated cell walls contained glucosamine and muramic acid in the ratio of approximately 1:1.

The finding that extracellular slime gave similar reactions with antisera against whole cells or slime suggested that the serologically active components in extracellular slime also occurred cell wall-associated and were released from cell walls. This was confirmed by the observation that identical antigens were demonstrated in acid extract and extracellular slime. These results corroborated the findings of BOWDEN and HARDIE [1973].

The role of extracellular slime of Ny1 in the formation of dental plaque might be fruitfully studied by means of labelled antibodies, and, therefore, it was attempted to prepare a suitable antiserum. However, it appeared that the extracellular antigens, against which precipitating antibodies could be produced, accounted for only a minor portion of the bulk of the material.

Acknowledgements

I should like to thank Miss MARIETTE V. D. HOF for valuable technical assistance and Mr. GEORGE BOWDEN for his contribution to this work and his valuable suggestions in the preparation of the manuscript.

References

BERGLUND, F. and SORBO, B.: Turbidimetric analysis of sulfate in serum, plasma and urine. Scand. J. clin. Lab. Invest. *12:* 147–153 (1960).

BOWDEN, G. H.: The components of the cell wall and extracellular slime or four strains of *Staphylococcus salivarius* isolated from human dental plaque. Arch. oral Biol. *14:* 685–697 (1969).

BOWDEN, G. H. and HARDIE, J. M.: Commensal and pathogenic *Actinomyces* species; in SYKES and SKINNER Actinomycetales, pp. 277–299 (Academic Press, New York 1973).

CARLSSON, J Presence of various types of non-haemolytic streptococci in dental plaque and in other sites of the oral cavity in man Odont Rev *15* 55–74 (1967)

CARLSSON, J Nutritional requirements of *Streptococcus mutans* Caries Res *4* 305–320 (1970)

CESSI, C and PILIEGO, F The determination of amino sugars in the presence of amino acids and glucose Biochem J *77* 508–510 (1960)

CHEN, P S, TORIBARA, T Y, and WARNER, H Microdetermination of phosphorus Analyt Chem *28* 1756 (1956)

DAWES, E A, MCGILL, D J, and MIDGLEY, M Analysis of fermentation products, in NORRIS and RIBBONS Methods in enzymology, vol 6A, p 123 (Academic Press, New York 1971)

DISCHE, Z A specific new color reaction of hexuronic acids J biol Chem *167* 189 (1947)

DISCHE, Z Colour reactions of hexoses, in WHISTLER and WOLFROM Methods in carbohydrate chemistry, vol 1, p 492 (Academic Press, New York 1962)

DUBOIS, M, GILLES, K A, HAMILTON, J K, REBERS, P A, and SMITH, F Colorimetric method for determination of sugars and related substances Analyt Chem *28* 350–356 (1956)

GERENCSER, M A and SLACK, J M Identification of human strains of *Actinomyces viscosus* Appl Microbiol *18* 80 87 (1969)

GUGGENHEIMM B Enzymatic hydrolysis and structure of water-insoluble glucan produced by glucosyltransferases from a strain of *Streptococcus mutans* Helv odont Acta *14* 88–100 (1970a)

GUGGENHEIM, B Extracellular polysaccharides and microbial plaque Int dent J *20* 657–678 (1970b)

HAMMOND, B F Studies on encapsulated lactobacilli III Human oral strains J dent Res *46* 340–346 (1967)

HAMMOND, B F, STEEL, C F, and PEINDL, K Occurrence of 6-deoxytalose in cell walls of plaque actinomycetes Abstr Papers 51st General Meet IADR, Washington 1973 J dent Res *52* 88 (1973)

HENKEL, H G Gas chromatographic analysis of low boiling fatty acids in biological media J Chromat *58* 201–207 (1971)

HESTRIN, S The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine, and its analytical application J biol Chem *180* 249–261 (1949)

HOEVEN, J S VAN DER, MIKX, F H M, KONIG, K G, and PLASSCHAERT, A J M Plaque formation and dental caries in gnotobiotic and SPF-Osborne-Mendel rats associated with *Actinomyces viscosus* Caries Res (in press, 1973)

HOWELL, A and JORDAN, H V A filamentous microorganism isolated from periodontal plaque in hamsters II Physiological and biochemical characteristics Sabourandia *3* 93–105 (1963)

HOWELL, A and JORDAN, H V Production of an extracellular levan by *Odontomyces viscosus* Arch oral Biol *12* 571–573 (1967)

JEANES, A, WISE, C S, and DIMLER, R J Improved techniques in paper chromatography of carbohydrates Analyt Chem *23* 415–421 (1951)

JORDAN, H. V. and KEYES, P. H.: Aerobic, gram-positive, filamentous bacteria as etiologic agents of experimental periodontal disease in hamsters. Arch. oral Biol. *9:* 401–414 (1964).

KEYES, P. H.; JORDAN, H. V.; HOWELL, A., and FITZGERALD, R.: A transmissible and infectious type of experimental periodontal disease. Program and Abstracts of Papers. Int. Ass. dent. Res. *1963:* 115.

KOCUR, M.; BERGAN, T., and MORTENSEN, N.: DNA base composition of gram positive cocci. J. gen. Microbiol. *69:* 167–183 (1971).

KRASSE, B.: Human streptococci and experimental caries in hamsters. Arch. oral Biol. *11:* 429–436 (1966).

KRASSE, B. and CARLSSON, J.: Various types of streptococci and experimental caries in hamsters. Arch. oral Biol. *15.* 25–32 (1970).

LOWRY, O. H.; ROSENBROUGH, N. J.; FARR, A. L., and RANDALL, R. J.: Protein measurement with the folin phenol reagent. J. biol. Chem. *193* 265–275 (1951).

MIKX, F. H. M.; HOEVEN, J. S. VAN DER; KÖNIG, K. G., and PLASSCHAERT, A. J. M.: Establishment and predominance of *Streptococcus mutans* and *Streptococcus sanguis* strains in rats on different sucrose diets. Caries Res. (in press, 1973).

PARKER, R. B. and CREAMER, H. R.: Contribution of plaque polysaccharides to growth of cariogenic microoganisms. Arch. oral Biol. *16:* 855–862 (1971).

PINE, L.: Classification and phylogenetic relationship of microaerophilic actinomyces. Int. J. syst. Bact. *20.* 445–474 (1970).

RITZ, H. L.: Localization of Nocardia in dental plaque by immunofluorescence. Proc. Soc. exp. Biol. Med. *113* 925–929 (1963).

SCHNEIDER, W. C.: Phosphorous compounds in animal tissues. III. A comparison of the methods for the estimation of nucleic acids. J. biol. Chem. *164* 747–751 (1946).

SPIRO, R. G.: in NEUFELD and GINSBURG Methods in enzymology, vol. 3, p. 6 (Academic Press, New York 1966).

STOPPELAAR, J. D. DE; HOUTE, J. VAN, and MOOR, C. E. DE: The presence of dextran-forming bacteria, resembling *S. bovis* and *S. sanguis*, in human dental plaque. Arch. oral Biol. *12:* 1199–1201 (1967).

STOPPELAAR, J. D. DE; HOUTE, J. VAN, and BACKER DIRKS, O.: The relationship between extracellular polysaccharide producing streptococci and smooth surface caries in 13-year-old children. Caries Res. *3* 190–199 (1969).

STOPPELAAR, J. D. DE; HOUTE, J. VAN, and BACKER DIRKS, O.: The effect of carbohydrate restriction on the presence of *Strep. mutans* and *Strep. sanguis* and iodophilic polysaccharide producing bacteria in human dental plaque. Caries Res. *4:* 114–123 (1970).

Request reprints from: J. S. VAN DER HOEVEN, Institute of Preventive Dentistry, University of Nijmegen, *Nijmegen* (The Netherlands)

Caries Res. 8 211–223 (1974)

IV

Plaque Formation and Dental Caries in Gnotobiotic and SPF Osborne-Mendel Rats Associated with *Actinomyces viscosus*

J. S. VAN DER HOEVEN, F H. M. MIKX, K. G. KÖNIG and A. J. M. PLASSCHAERT

Institute of Preventive and Community Dentistry, University of Nijmegen, Nijmegen

Abstract The slime-producing microorganism *A viscosus* Ny1 was tested on its plaque-forming ability and its cariogenicity in rats fed on glucose or sucrose diets. In mono-associated as well as SPF O-M rats, strain Ny1 caused considerable plaque formation on both diets The role of the resident microflora on the establishment of strain Ny1 was investigated by varying its composition with antibiotics It was found that pre-experimental treatment with erythromycin prevented the establishment of strain Ny1 in SPF rats In mono-associated rats, strain Ny1 caused no smooth surface caries and little fissure caries However, in SPF rats, the interaction of strain Ny1 with the resident microflora resulted in considerable smooth surface and fissure caries on both diets Smooth surface caries was greater on the sucrose diet.

Key Words
A viscosus
Caries in rats
Glucose diet
Plaque formation
Sucrose diet

Strains of *Actinomyces viscosus* (serotype 1) have been isolated in the course of screening bacteria that accumulate in the dental plaque of rats kept on a high-glucose diet [VAN DER HOEVEN, 1974]. One of the isolates, strain Ny1, has subsequently been tested on its plaque-forming ability and potential cariogenicity in mono-associated rats. The effect of glucose or sucrose as main carbohydrate constituents of the diet has been compared. In another experiment, the implantation of strain Ny1 has been studied in specific pathogen-free (SPF) rats. It was shown by KRASSE [1966] that the establishment and prevalence of a *Strep mutans* strain in hamsters was governed by the presence of sucrose in the diet. The implantation of

The technical assistance of Mr JAN LUKASSEN and Mr JAN KOEDAM is gratefully acknowledged

Strep. sanguis strains, however, failed under similar conditions [KRASSE and CARLSSON, 1970]. It was suggested that this might be ascribed to species-specific factors but also to the influence of the resident microflora. In the present experiment, an attempt was made to investigate the role of the resident microflora by varying its composition with antibiotics. The experiment included two periods, during the first of which a glucose diet was administered. The effect of sucrose on the occurrence of Ny1 in plaque was investigated in the second period.

Materials and Methods

Microorganisms

A viscosus was found to accumulate in dental plaque of Osborne-Mendel rats kept on a high-glucose diet *A viscosus* strain Ny1 has been isolated from one of the animals and this strain was tested in the present experiments Characteristics of the strain have been described [VAN DER HOEVEN, 1974] In liquid cultures containing glucose, strain Ny1 synthesizes viscous polymer which can be isolated from the medium by solvent precipitation

In the presence of sucrose, Ny1 produces extracellular fructan Strain Ny1 was found to be fairly resistent to streptomycin (1 mg/ml) and sensitive to erythromycin (10 μg/ml) when tested in broth cultures

Inoculation

The inoculum was prepared by growing strain Ny1 in actinomyces broth (BBL) 24 h at 37 °C The suspension was applied to each rat with a cotton stick

Bacteriological Checks

In both experiments, samples from the oral cavity of 2 rats from each treatment group were taken weekly with cotton sticks; plaque samples were taken with a dental scaler after sacrificing the rats. All samples were homogenized in 5 ml transport medium [DE STOPPELAAR *et al*, 1969] and diluted in saline Suitable dilutions were plated on blood agar and incubated 72 h at 37 °C in an atmosphere of 90% N_2 and 10% CO_2 In plaque samples from SPF rats, different colony types were distinguished under the dissecting microscope and Gram stains were made of each colony type. The number of Ny1 colonies in every sample was expressed as percentage of viable counts on blood agar. The SPF microflora contained only a limited number of species and, therefore, colonies of strain Ny1 on blood agar could be easily recognized in the samples

Animals and Treatments

A. viscosus strains Ny1 was tested in gnotobiotic and SPF Osborne-Mendel rats.

Mono-associated rats Germ-free rats (10) from 4 litters weaned at 24 days were individually marked and distributed at random among 2 treatment groups. Each group consisted of 3 rats from 3 litters and 2 rats from a 4th litter. Both treatments

Table I Composition of diets 540 G, 540 S, 2000 G and 2000 S

	540 G %	540 S %	2000 G %	2000 S %
Glucose	40	–	56	–
Sucrose	–	40	–	56
Skim milk powder	32	32	28	28
Wheat flower (70% extr.)	21	21	8	8
Brewers yeast[1]	5	5	7	7
Gevral protein[2]	2	2	–	–
NaCl	–	–	1	1
Vitamin mixture[3]	+	+	–	–

[1] Brewers yeast 'Engevita'
[2] Vitamin protein mineral supplement Lederle
[3] According to Gustafsson's diet D7 [Luckey 1963, p. 494]

were carried out in the same isolator with 2 cages per group, each cage containing 2 or 3 rats. The treatments consisted in the administration of a glucose diet (540 G) or a sucrose diet (540 S) (table I), for an experimental period of 30 days. All animals were individually inoculated with *A. viscosus* strain Ny1. At the end of the experimental period, the rats were weighed.

SPF rats. In this experiment, 144 SPF rats, originating from 26 litters, were weaned at an age of 22–26 days, they were individually marked and distributed at random among 8 groups (table II). The rats were housed in stainless steel cages each containing 3 rats. The resident SPF flora was suppressed with erythromycin lactobionate (Abbott) or streptomycin sulphate (Sigma) or both antibiotics. The antibiotics were supplied in the drinking water (2 g/l) for 6 days prior to inoculation. The rats in groups 1–4 were inoculated with strain Ny1 at days 7, 8 and 11. During the first experimental period of 29 days, the rats were fed diet 200 G *ad libitum*. After this period, 48 rats, one rat from each cage (6 per treatment), were sacrificed. Half of the remaining number of rats were changed to a sucrose diet 2000 S (b), the other half remained on the glucose diet (a) (table II). At the end of the second experimental period (25 days), all rats were sacrificed. The animals were weighed after weaning and at the end of each experimental period.

Diets. In both experiments, rats were fed *ad libitum* low fat and high carbohydrate diets (table I). The gnotobiotic rats were maintained on diet 540 G and 540 S. These diets had been sterilized by γ-irradiation 2.5 Mrad. The SPF rats were fed on diets 2000 G and 2000 S.

Evaluation of plaque and caries. After sacrifice, the jaws were removed and fixed in 10% buffered formalin. Plaque was stained with 0.1% brilliant green (Fluka). Plaque accumulation [Konig, 1959], smooth surface caries [Keyes, 1958] and fissure lesions [Konig, 1966] were scored using the same methods as in previous investigations [van der Hoeven *et al.*, 1972].

Table II. Arrangement of treatments and groups[1]

Treatment		Groups							
		1	2	3	4	5	6	7	8
Erythromycin, 2 g/l		−	−	+	+	−	−	+	+
Streptomycin, 2 g/l		−	+	−	+	−	+	−	+
Strain Ny1		+	+	+	+	−	−	−	−
Period 1 (29 days)									
Diet 2000 G									
Number of rats	(a)	18	18	18	18	18	18	18	18
Period 2 (25 days)									
Diet 2000 G									
Number of rats	(b)	6	6	6	6	6	6	6	6
Diet 2000 S									
Number of rats	(c)	6	6	6	6	6	6	6	6

[1] Association of SPF rats with *A. viscosus* strain Ny1. Antibiotics were administered during a 6-day period before inoculation. After 29 days, one third of the animals, 6 out of each treatment, were sacrificed (treatments a). The remaining animals of each treatment were subdivided into 2 groups, one receiving diet 2000 G (treatments b) and the other diet 2000 S (treatments c).

Results

Bacteriological Findings in Mono-Associated Rats

In both dietary groups of rats, strain Ny1 was present in the mouth as a monocontaminant during the whole experimental period. Strain Ny1 occurred only incidentally in the feces and was found to be virtually absent in the coecum at the end of the experiment.

Bacteriological Findings in SPF Rats

The establishment of Ny1 was found to be influenced by the pre-experimental treatment of the animals with antibiotics. Ny1 could be recovered in high numbers from non-treated (group 1) and streptomycin-treated animals (group 2). The presence of Ny1 in plaque could not be demonstrated when inoculated after application of erythromycin (groups 2, 4). The percentages of Ny1 of total viable counts in plaque showed increasing variation and was found to decrease at the end of the

Table III. Presence of Ny1 in plaque expressed as mean percentage of total viable counts[1]

	A. viscosus Ny1 (percentage of total viable count)	
	2000 G diet	2000 S diet
First experimental period	75 (60–85)[2]	–
Second experimental period	42 (11–86)	44 (22–65)

[1] Plaque was sampled from 2 rats taken from each glucose (1a, 2a, 1b, 2b) and sucrose (1c, 2c) group. Ny1 was not found in other groups.

[2] Range.

Table IV. Plaque and caries scores, and final weights in two groups of 5 rats, mono-associated with *A viscosus* strain Ny1[1]

	Diet	
	glucose (540 G)	sucrose (540 S)
Plaque	2.0±0.7	2.0±1.0
Fissure caries		
All lesions (A+T)	2.0+2.3	1.8±1.3
Dentinal lesions (T)	0.4±0.5	0.2±0.4
Final weight, g	197.0 (♂=3)	196.2 (♂=4)

No smooth surface lesions were observed.

[1] Averages of plaque accumulation in lower jaws and of fissure lesions in first and second molars of lower jaws.

second period (table III). Virtually, no influence could be observed of the kind of dietary sugar on the relative amount of Ny1 in plaque.

Plaque Findings in Mono-Associated Rats

Considerable plaque formation was induced by Ny1. Plaque was formed to the same extend in both dietary groups of rats (table IV).

Plaque Findings in SPF Rats

The results of the evaluation of plaque after the first (29 days) experimental period are presented in table V and figure 1a. Thick plaque was only found in treatments 1 and 2 where Ny1 could be established. Little

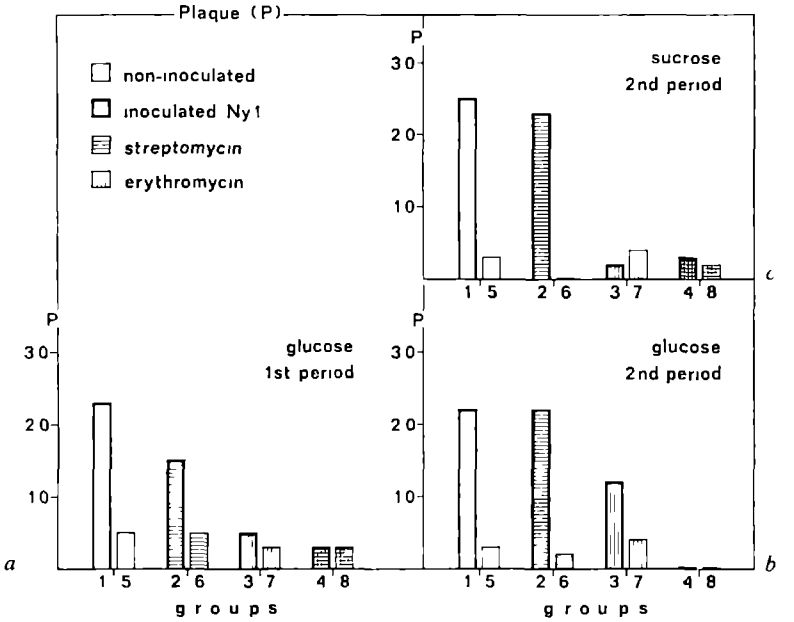


Fig. 1. Extent of smooth surface plaque (P) in 8 groups of SPF Osborne-Mendel rats treated with antibiotics, inoculated with *A. viscosus* Ny1 and fed on different diets, for varying experimental periods: *a* 29 days on glucose *b* 54 days on glucose. *c* 54 days on sucrose.

plaque was found in the non-inoculated control animals and in the animals treated with erythromycin. Similar results were obtained at the end of the second period (table VI; fig. 1b, c). Thick plaque was only observed in groups 1 and 2. No difference was found between the glucose- and sucrose-fed animals. A slightly increased plaque score was found in the erythromycin-treated glucose-fed animals (group 3). This was found to be due to a high score for 2 animals in this treatment. In these animals, Ny1 was shown to occur in the plaque, although Ny1 was not observed in oral samples taken during the experiment.

Caries Findings in Mono-Associated Rats

A low number of fissure lesions was found in both dietary groups of rats (table IV). No smooth surface lesions were observed at the end of the 30-day experimental period.

Table V Plaque extent, smooth surface carious units, dentinal fissure lesions and weight gains in SPF rats associated with *A viscosus* Ny1[1]

Group number	Antibiotic treatment	Establishment of Ny1	Plaque	Smooth surface lesions	Fissure lesions	Weight gains
Inoculated Ny1						
1	–	+	2.3±0.5	1 0	6 ±4.1	103 (3)[2]
2	str	+	1 5±0.9	0	2 5±1.9	117 (4)
3	ery	–	0 5±0 5	0	1.8±1.6	102 (3)
4	ery+str	–	0 3±0 5	0	1 8±1 6	112 (2)
Controls						
5	–	–	0.5±0 5	0	3 ±2 4	95 (3)
6	str	–	0 5±0 8	0	3 0±1.7	104 (3)
7	ery	–	0.3±0.5	0	3 ±2.5	109 (2)
8	ery+str	–	0 3±0.5	0	2.2±1.9	100 (2)

[1] Results of the first 29-day period. Diet 2000 G (glucose) Averages and standard deviations for 8 groups of 6 rats. ery = erythromycin; str = streptomycin; administration before inoculation during 6 days in drinking water.

[2] Number of males in parentheses.

Caries Findings in SPF Rats

Smooth surface caries. Smooth surface caries scores at the end of the first experimental period (22 days after inoculation) are shown in table V and figure 3a. Virtually, no smooth surface caries was found, except a very low number of lesions in group 1.

The distribution of smooth surface lesions after the second period (47 days after inoculation) is presented in table VI and figure 3b, c. The control groups showed very little smooth surface lesions. A high number of lesions were found in those rats in which Ny1 could be established (groups 1, 2). The number of lesions in the sucrose groups was found to be significantly higher than in the respective glucose groups especially in the streptomycin-treated animals (fig. 3b, c).

Fissure caries. Fissure lesions at the end of the first period are shown in table V and figure 2. A tendency towards higher caries activity due to the establishment of *A. viscosus* Ny1 was found in group 1, but not in group 2 where Ny1 was also established. The distribution of fissure le-

Table VI. Plaque extent, smooth surface carious units, dentinal fissure lesions and weight gains in SPF rats associated with *A. viscosus* Ny[1]

Group number	Antibiotic treatment	Establishment of Ny1	Plaque		Smooth surface		Fissures		Weight gain	
			g	s	g	s	g	s	g	s
Inoculated Ny1										
1	–	+	2.2±0.7	2.5±0.5	14 ±4.5	23 ±8.0	10.2±1.1	8.8±1.9	198 (5)[2]	186 (3)[2]
2	str	+	2.2±0.7	2.3±0.5	4 ±5.8	22 ±5.0	5 ±2.1	7.0±1.5	169 (2)	195 (4)
3	ery	–	1.2±1.2	0.2±0.4	0.5±1.1	0.0	3 ±2.3	2.7±1.8	186 (4)	173 (3)
4	ery+str	–	0.0	0.3±0.7	0.0	0.0	3.3±1.1	3 ±2.0	183 (3)	150 (1)
Controls										
5	–	–	0.3±0.5	0.3±0.5	0.7±1.5	0.0	5 ±2.1	2 ±2.2	132 (0)	168 (2)
6	str	–	0.2±0.4	0.0	0.5±1.1	0.2±0.4	4.3±1.7	3 ±2.0	178 (3)	178 (3)
7	ery	–	0.4±0.5	0.4±0.5	0.0	0.0	2.6±1.5	2.7±1.7	187 (4)	182 (3)
8	ery+str	–	0.0	0.2±0.4	0.0	0.0	1.7±0.9	1.4±1.2	170 (2)	168 (3)

[1] Experimental period 54 days; diets 2000 G (g), 2000 S (s). Averages and standard deviations (n = 6 rats per group). Drugs were administrated before inoculation during 6 days in drinking water. ery = erythromycin; str = streptomycin.

[2] Number of males in parentheses.

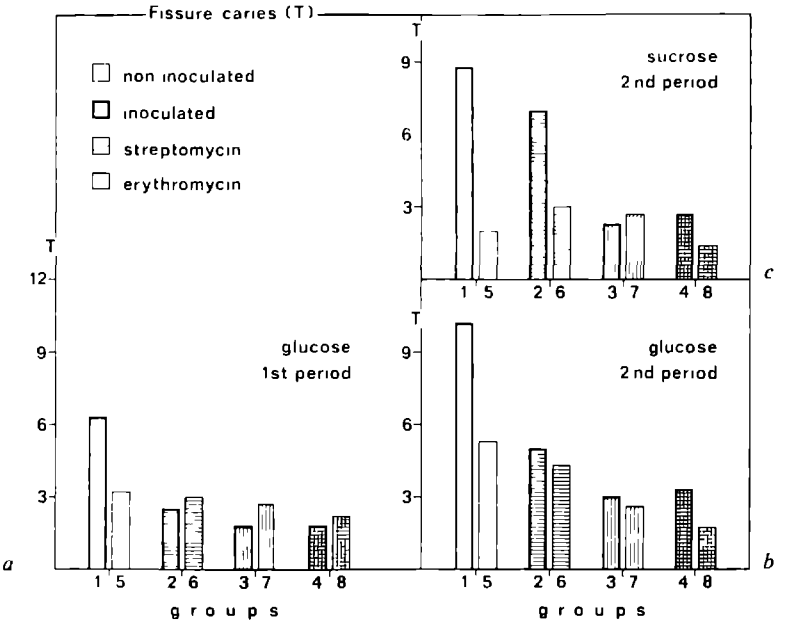


Fig. 2. Fissure caries incidence (T) in 8 groups of SPF Osborne-Mendel rats treated with antibiotics, inoculated with *A. viscosus* Ny1 and fed on different diets, for varying experimental periods: *a* 29 days on glucose. *b* 54 days on glucose. *c* 54 days on sucrose.

sions on prolonging of the experiment is shown in table VI and figure 2b, c.

The number of dentinal fissure lesions was significantly increased by establishment of Ny1, except in the glucose-fed streptomycin-treated animals (fig. 2b). A significantly higher number of fissure lesions was found in the non-treated glucose-fed animals, than in the respective sucrose groups (compare groups 5 in fig. 2b, c).

The number of lesions in the control groups was found to be only slightly increased by continuation of the experimental period from 22 to 47 days (groups 5, 6, 7, 8 in fig. 2a–c).

Growth of the Animals

Growth of the animals was normal in both experiments on all treatments, as judged from terminal average weights (tables IV–VI).

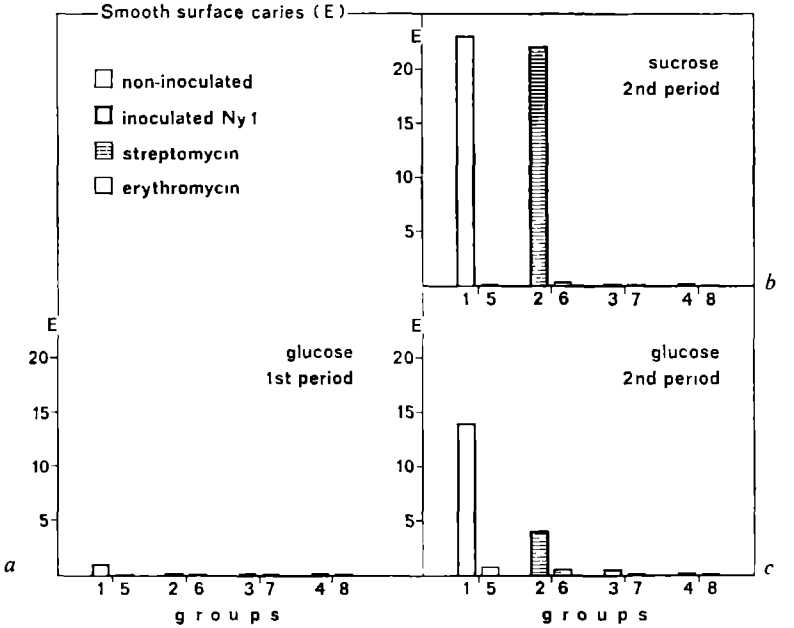


Fig. 3. Smooth surface caries incidence (E) in 8 groups of SPF Osborne-Mendel rats treated with antibiotics, inoculated with *A. viscosus* Ny1 and fed on different diets, for varying experimental periods. a 29 days on glucose. b 54 days on glucose. *c* 54 days on sucrose.

Discussion

Strain Ny1 was found to be weakly cariogenic in mono-associated rats, and plaque was produced to the same extent on glucose and sucrose diets. Similar results were obtained by LLORY *et al.* [1971] who tested a human strain of *A. viscosus* in mono-associated rats on sucrose diet. In both investigations, there is a discrepancy between the high plaque score and the low caries scores. This might be due to the relatively low production of lactic acid by strain Ny1 when abundant hexose substrate is available, but relatively little protein [VAN DER HOEVEN, 1973]. Such a situation is likely to occur in dental plaque [HOTZ *et al.*, 1972; VAN HOUTE and SAXTON, 1971].

The occurrence of smooth surface lesions in SPF-rats was found to be strictly correlated to the presence of strain Ny1 in plaque. Virtually, no lesions had developed during an experimental period of 22 days after in-

oculation Prolonging the experiment by 25 days resulted in considerably more lesions

Therefore, the absence of smooth surface lesions in mono-associated rats could be due to the relatively short experimental period of 30 days In subsequent tests, however, it has been shown that prolongating the experimental period up to 42 days did not result in more smooth surface lesions

A viscosus was found to produce extracellular fructan from sucrose [HOWELL and JORDAN, 1967] The observation that thick plaque was also formed by actinomyces in the absence of sucrose and levan synthesis, suggests that polymers other than levan are involved It might be that the extracellular polymer which is synthesized by Ny1 in glucose broth [VAN DER HOEVEN, 1973] functions as matrix substance

When Ny1 was inoculated in animals harbouring an indigenous (SPF) microflora, it was found that pre-experimental treatment with erythromycin prevents the establishment of the strain This could be due to inhibition of Ny1 by traces of the drug left in the oral cavity However, it was observed that blood or saliva taken from the animals 24 h after administration of the drug did not show any inhibitory action on the growth of Ny1 on agar media Furthermore, Ny1 could be easily established in germ-free animals treated with erythromycin and inoculated according to a similar time schedule as the SPF rats The failure of strain Ny1 to establish in erythromycin-treated rats may be caused by an increased resistency to colonization of the remaining flora This emphasizes the important role of the resident flora to establishment of orally introduced microorganisms

A significant increase in smooth surface caries incidence was observed when Ny1 was successfully implanted (table VI, fig 3a–c) The increased cariogenicity might be explained merely by the increase in plaque thickness induced by Ny1 However, this cannot explain the significant difference in cariogenicity of the plaque in sucrose- and glucose-fed animals (groups 2 in fig 3b, c) since plaque score and relative amount of Ny1 were found to be similar in both groups (table III) Therefore, it seems that sucrose influences the interaction of the SPF flora with Ny1, thereby increasing the cariogenicity of the smooth surface plaque more than glucose

Streptomycin could have eliminated those components of the flora which are responsible for the caries activity with glucose but not with sucrose This corroborates the results for fissure caries where it was also

found that the caries activity was decreased by streptomycin in the glucose-fed animals but not in the sucrose-fed animals (groups 2 in fig. 2b, c).

An attempt was made to compare the microbial composition of plaque formed on different treatments. A particular type of colony, resembling *Strep. sanguis* was found to occur exclusively when Ny1 was simultaneously present in plaque. Many other relations may have been overlooked since discrimination of species based on colonial morphology and Gram stain has only limited validity. The significance for the cariogenicity of the plaque of the relationship between Ny1 and this particular type of *Strep. sanguis* will be further investigated in gnotobiotic rats di-associated with both strains.

JORDAN and KEYES [1965] and JORDAN *et al.* [1972] observed that *A. viscosus* causes severe periodontal lesions, alveolar bone loss and root caries in hamsters and mono-associated rats. In the present experiment, soft tissue recession and pocket formation were observed in some gnotobiotic and SPF rats associated with Ny1. The less severe character of these lesions might be partly attributed to the relatively short experimental period compared to the experiments cited. No further attempts were made to evaluate these observations.

References

HOEVEN, J S VAN DER. A slime producing microorganism in dental plaque of rats, selected by glucose feeding. Chemical composition of extracellular slime elaborated by *Actinomyces viscosus* strain Ny1. Caries Res *8* 193–210 (1974)

HOEVEN, J S. VAN DER; MIKX, F. H. M ; PLASSCHAERT, A J M , and KONIG, K G Methodological aspects of gnotobiotic caries experimentation. Caries Res *6* 203–210 (1972).

HOTZ, P , GUGGENHEIM, B., and SCHMID, R : Carbohydrates in pooled dental plaque Caries Res *6*· 103–121 (1972)

HOUTE, J. VAN and SAXTON, C. A : Cell wall thickening and intracellular polysaccharide in microorganisms of dental plaque. Caries Res *5* 30–43 (1971).

HOWELL, A., jr. and JORDAN, H V Production of an extracellular levan by *Odontomyces viscosus*. Arch. oral Biol. *12* 571–573 (1967)

JORDAN, H. V. and KEYES, P. H.· Studies in the bacteriology of hamsters periodontal disease Amer J Path *46* 843–857 (1965).

JORDAN, H. V.; KEYES, P. H., and BELLACK, S Periodontal lesions in hamsters and gnotobiotic rats infected with actinomyces of human origin J Periodont Res *7* 21–28 (1972).

KEYES, P. H Dental caries in the molar teeth of rats. II. A method for diagnosing and scoring several types of lesions simultaneously. J. dent. Res. *37* 1088–1099 (1958).

KONIG, K G Dental caries and plaque accumulation in rats treated with stannous fluoride and penicillin Helv. odont Acta *3* 39–44 (1959)

KONIG, K G Moglichkeiten der Kariesprophylaxe beim Menschen und ihre Untersuchungen im kurzfristigen Rattenexperiment, pp 67–77 (Huber, Bern 1966).

KRASSE, B Human streptococci and experimental caries in hamsters Arch oral Biol *11* 429–436 (1966)

KRASSE, B and CARLSSON, J Various types of streptococci and experimental caries in hamsters Arch oral Biol *15* 25–32, 1970

LLORY, H , GUILLO, B , and FRANK, R M A cariogenic *Actinomyces vicsosus* A Bacteriological and gnotobiotic study Helv odont Acta *15* 134 (1971)

LUCKEY, T D Germfree life and gnotobiology (Academic Press, 1963)

STOPPELAAR, J D DE, HOUTE, J VAN, and BACKER DIRKS, O The relationship between extracellular polysaccharide-producing streptococci and smooth surface caries in 13-year old children Caries Res *3* 190–199 (1969)

Request reprints from J S VAN DER HOEVEN, Institute of Preventive and Community Dentistry, University of Nijmegen, *Nijmegen* (The Netherlands)

EXPERIMENTAL PERIODONTAL DISEASE IN RATS INDUCED BY PLAQUE-FORMING MICROORGANISMS THAT WERE SELECTED BY GLUCOSE FEEDING*

J.S. VAN DER HOEVEN, F.H.M. MIKX AND A.J.M. PLASSCHAERT

Institute of Preventive and Community Dentistry
University of Nijmegen

and

J.C. MALTHA

Laboratory of Oral Histology
University of Nijmegen

* Presented for publication in Journal of periodontal Research

Summary

The three different types of microorganisms, that were tested in this study, originated from a child (*Rothia* sp. Ny21) or from beagle dogs (*Neisseria* sp. Ny204 and *Bacterionema* sp. Ny205).

Selection was based upon the ability of the microorganisms to produce extracellular polymer as judged by indian ink preparations. All three test strains were found to stimulate plaque formation in SPF-rats fed on a glucose diet. The histological findings in the rats suggested that each of the strains contributed to the initiation of periodontal disease.

Introduction

Glucose feeding of rats resulted in the accumulation of *Actinomyces viscosus* serotype 1 in dental plaque, furthermore, *A viscosus* stimulates plaque formation in glucose-fed animals possibly by virtue of its ability to synthesize extracellular slime (v.d. Hoeven 1974, v.d. Hoeven *et al.* 1974) Although *A. viscosus* may be an important organism forming slime from glucose, other bacterial species with similar characteristics may occur in plaque, and attempts were made to isolate such organisms from animal and human dental plaque.

Two organisms Ny 204 and Ny 205 were isolated from plaque formed in rats which had been orally inoculated with plaque from beagle dogs that had severe clinical periodontitis. These rats were maintained on a glucose diet. The third strain (Ny 21) was isolated from samples of plaque from a child given a diet containing glucose in place of sucrose. This paper describes tests in rats which were aimed at determining the possible role of the isolated microorganisms in the development of periodontal disease.

Methods

Selection of microorganisms

The 3 different strains tested in this study were isolated from a child (strain Ny 21) or originated from beagle dogs (strains Ny 204 and Ny 205). Selection was based upon the ability of the organisms to produce extracellular material as judged by indian ink preparations. Details concerning the isolation of these strains are described below.

Strain Ny 21. A two year old boy (the son of J.S. v.d. H.) was fed on a diet without sucrose. Glucose however was given frequently in all kinds of foodstuffs which normally contain sucrose. The glucose regime was continued for about 5 months. At monthly intervals, plaque samples were removed from the buccal of the maxillary first deciduous molars. Plaque was homogenized and cultured on bloodagar and brain-heart infusion agar with 1% w/v glucose. A Gram positive encapsulated filamentous rod was regularly isolated from the plaque in high numbers during the glucose regimen, Ny 21 represents one strain of these organisms.

Strains Ny 204 and Ny 205. Sub- and supra gingival plaque was collected from 2 beagle dogs showing the clinical signs of severe periodontitis. The pooled plaque was immediately transferred into transport medium (de Stoppelaar *et al.* 1969) homogenized on a Vortex mixer and stored at 4°C, the storage period did not exceed 2 h. A test group of 24 young SPF Osborne-Mendel rats were inoculated with the pooled homogenized plaque. A non inoculated control group of animals was included in the series. The rats were fed the glucose diet 516 G containing 16% glucose, 45% wheat flour (70% extraction) 32% skim milk and 7% brewers yeast. Sixty days after inoculation the animals were sacrified and samples of plaque were removed and cultured as above. Strains Ny 204 and Ny 205 were isolated from the inoculated rats, no similar organisms were present in the control group. The three isolated strains (Ny 21, Ny 204 and Ny 205) were kindly examined by G.H. Bowden (The London Hospital, Dental School). Ny 21 was identified as a member of the genus *Rothia*, Ny 204 a *Neisseria* sp. and Ny 205 a *Bacterionema* sp.

Measurement of the activities of the isolates in vivo

Strains Ny 21, Ny 204 and Ny 205 were tested regarding their effect on plaque formation and their ability to induce periodontal disease in SPF-Osborne-Mendel rats.

Table I Summary of taxonomic criteria tested on 3 different isolates

Test or characteristic	strains		
	Ny 21	Ny 204	Ny 205
growth on bloodagar aerob	+	+	+
anaerob	+	+	+
growth on starch agar aerob	+	+	+
anaerob	+	+	+
Gram stain	positive	negative	positive
cellular morphology	branched filamentous	coccoid	branched filamentous
presence of capsule or slime	capsule	slime	slime
fermentations	pH	pH	pH
glucose	5 0	5 3	4 6
lactose	6 4	6 8	6 3
sucrose	4 5	6 7	4 6
salicin	6 4	7 2	6 5
fructose	5 7	6 7	5 0
xylose	6 5	6 6	6 5
arabinose	6 4	6 3	6 3
raffinose	6 6	6 9	6 6
mannitol	6 7	6 7	6 6
cellobiose	6 5	6 4	6 2
trehalose	4 9	6 7	4 6
glycerol	6 3	6 7	6 2
amygdalin	6 2	6 0	6 1
inositol	6 7	6 8	6 6
Voges Proskauer	n t	n t	+
H_2S	–	–	–
aesculin		–	–
indole	–	–	–
nitrate	+	±	–
nitrite	+	–	–
starch hydrolysis	–		
catalase	+	+	+
oxidase	n t	+	n t
fermentation products			
acetic acid	+	n t	+
propionic acid	–	n t	+
lactic acid	+	n t	+
cell wall components			
diabasic amino acids	lysine	n t	DL DAP
carbohydrates	galactose	n t	galactose arabinose
diagnosis	*Rothia* sp	*Neisseria* sp	*Bacterionema* sp

n t = not tested
Examination of the strains was done by G H Bowden (The London Hospital Dental School)

Animals and treatments

At the age of 26 ± 2 days, 5 blocks of 8 littermates were formed, marked and distributed at random among 4 groups The rats were inoculated as follows

group 1 strain Ny 21
group 2 strain Ny 204
group 3 strain Ny 205
group 4 non-inoculated control

All rats were fed on diet 516 G, diet and tap water were available ad libitum The animals were housed in stainless steel cages with wire mesh bottoms without bedding

Inoculation

The inocula consisted of 24 h cultures of the respective strains in actinomyces broth (BBL) supplied with horse serum 20% (V/V) in 5 ml screw cap vials At 2 successive days the rats were individually inoculated by means of cotton sticks

Evaluation of plaque, caries and gingival tissue reaction

Ninety days after the first inoculation the animals were sacrificed and plaque samples were removed from the lower jaws, and cultured for the presence of the inoculated strains

The accumulation of plaque in the upper jaws and the occurrence of smooth surface and fissure caries in the molars of the lower jaw were scored according to methods described by Konig (1959), Keyes (1958) and Koning (1966) respectively

From each group 5 rats originating from the different litters were taken for histological evaluation

After fixation and scoring of plaque accumulation, the upper jaws were split in a midsagittal plane and then decalcified in 20% formic acid and 5% sodium citrate The halves were embedded in paraffin and 7 μ sections were prepared sagitally or frontally

Two parameters were choosen for assessment of the inflammatory reaction of the gingiva area infiltrated by inflammatory cells and 'total size' (refer to fig 1) as an indication for edema and/or hyperplasia

The amount of infiltrated tissue was scored in mesial sections of the buccal gingivae of the maxillary first right molars between the frontal and the central fissure (approx 20 sections of 7 μ) These sections were alternatively stained with either haematoxylin and eosin (HE) or with the van Gieson stain

From each rat 5 HE and 5 van Gieson stained sections were selected at random for evaluation.

In the HE stained sections the total size of the gingiva and the area of the infiltrated tissue was measured (Fig. 1). In the sections stained according to van Gieson the total gingival size and the area of collagen-poor, cell-rich tissue was determined. The measurements were done with a planimeter on drawings made of projections of the sections enlarged approximately 300 times. According to the magnified drawings the areas were given in cm^2. In order to investigate the differences between the variables with regard to both staining methods and inoculations, analyses of variance were applied on the data derived from the HE- and van Gieson-stained sections of the 4 groups for the infiltrated gingival tissue and the total gingival area respectively.

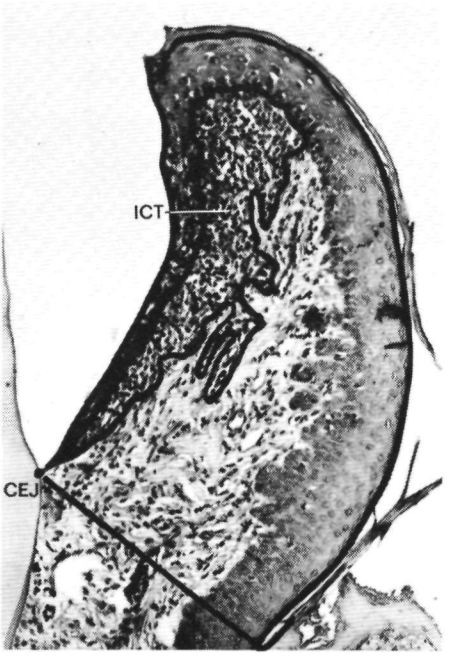


Fig. 1 HE-stained selection of buccal gingiva indicating how the measurements of total gingival area and infiltrated tissue were done. CEI = cemento junction. ICT = infiltrated connective tissue. Enlargement: 200 x.

Activities of the isolated strains in an animal experiment

Bacteriological findings

The presence of the inoculated strains at the end of the experiment could be demonstrated. There were no indications that cross contamination had occurred.

Caries incidence

The results of the evaluation for fissure caries are given in Table II. No significant differences were found in the average number of fissure lesions between the 4 groups. Smooth surface caries was not observed in any of the groups.

Table II Averages ± standard deviations of plaque score and dentinal fissure lesions (N = 10 rats) and, area (cm^2) of infiltrated gingival tissue and percentage of infiltrated gingival tissue (N = 5 rats) in 4 groups of SPF rats on diet 516 G. Group 1 *Rothia* sp. Ny 21, Group 2 *Neisseria* sp. Ny 204, Group 3: *Bacterionema* sp. Ny 205, Group 4. control

Group strain	1 Ny 21	2 Ny 204	3 Ny 205	4 –
	M ± SD	M ± SD	M ± SD	M ± SD
plaque	1.7 ± 0 6[2]	2.0 ± 0.4[3]	1.4 ± 0.6[3]	0.5 ± 0.5
caries	7.9 ± 2.4	8.5 ± 2.7	5 5 ± 2.5	6.0 ± 1.7
infiltrated	10.2 ± 7.4[1]	22.5 ± 9.5[3]	15.1 ± 7.6[3]	2.9 ± 4.9
%infiltrated	6.5 ± 3.0[2]	9.6 ± 4.6[3]	7.6 ± 3.5[3]	1.6 ± 2.8

No smooth surface caries was observed.

[1] $P_t < 0.05$ [2] $P_t < 0.01$ [3] $P_t < 0.01$

Plaque and gingival inflammation

The result of the evaluation of plaque is given in Table II. Significantly more plaque was found in the groups inoculated with the test strains Ny 21, Ny 204 and Ny 205, than in the non-inoculated control group.

The inflammatory response was evaluated on the buccal gingivae of the upper first molars. No assessment was done interdentally because here the effect of the experimental inflammatory agents was masked by hair impaction.

The average values for the amount of infiltrated tissue in HE-stained sections, expressed either in cm^2 (in 300 times enlarged projections) or as percentage of the total gingival size are presented in Table II.

As to infiltration of the gingiva both area and percentage of infiltrated tissue were found to be significantly larger in the inoculated groups than in the control group.

Analysis of variance (Table III) showed that the area of infiltrated tissue in the HE-stained sections was not significantly different from the collagen-poor cell-rich tissue in the van Gieson-stained sections.

With respect to the total size of the gingivae no significant differences were found between the 4 inoculation groups or between the two staining methods (Table III).

Table III Analyses of variance of area of infiltrated gingival tissue and of total gingival area encountered in 4 groups of 5 rats. DF = degrees of freedom; SS = sum of squares; MS = mean square.

		Infiltrate		Total gingival area	
Source of variance	DF	SS	MS	SS	MS
Between treatments	3	2036.97	678.99[1]	4198.86	1399.62
Stains	1	7.14	7.14	6340.09	6340.09
Interaction	3	0.32	0.11	8338.51	2779.50
Remainder	32	1858.48	58.08	94954.23	2967.32
Total	39	3902.91		113831.69	

[1] $P_F < 0.001$

Discussion

The experiments described are a continuation of a study to isolate encapsulated or slime-producing microorganisms from dental plaque and to assess their plaque-forming ability and pathogenic potential The change of the infant diet increased the relative proportion of *Rothia* in plaque due to replacement of sucrose by glucose The explanation may be a promoting effect of glucose on *Rothia* but in addition, ample supply of glucose may be inhibitory to other species thus increasing the effect on *Rothia* However, the lack of data on the occurrence of other species makes further conclusions impossible The constancy of the proportion of *Rothia* in plaque samples taken off during the glucose regime is similar to results of Bowden and Hardie (1973) who observed constant proportions in the course of time with respect to a number of different species in plaque removed from a localized area

The *Neisseria* and *Bacterionema* species were isolated only from the group of rats inoculated with the plaque from beagle dogs Therefore it seems likely that *Neisseria* and *Bacterionema* originated from the microflora of the beagle dog

The ability of *Neisseria* species to produce extracellular slime in the absence of sucrose was earlier reported by Parker and Creamer (1971)

It is noted that in the present animal experiment the increased plaque formation in the inoculated groups was not associated with an increase in cariogenicity That glucose was the dietary sugar underlines its potential significance in the formation of dental plaque All three test strains, Ny 21, Ny 204 and Ny 205 were selected on the basis of their ability to produce some kind of extracellular slime as revealed in wet-indian ink Up to date, no further investigation to the characteristics of the slime production or to the chemical nature of the slimes has been made

That the production of these extracellular slimes is involved in the formation of plaque can only be speculated but seems difficult to demonstrate

The histological findings in SPF rats suggest that each of the 3 test strains contribute to the initiation of periodontal inflammation These results are in accordance with the conclusion of Socransky (1970) that the ability to form dental plaque appears to be a prerequisite of all the organisms which have been shown to induce experimental periodontal disease

The relationship between accumulation of plaque and the initiation of periodontal disease was earlier established in clinical studies by Theilade *et al* , (1966) It is noteworthy that up to now it has not been possible to indicate

the etiologic role of plaque more precisely, neither with regard to the microbial composition nor to the chemical nature of the etiologic agents released.

Acknowledgements

The authors would like to express their thank to Mr. George Bowden (The London Hospital), for identification of the microorganisms and for valuable suggestions in the preparation of the manuscript, further to Miss Elly Lurvink and Mr. Jan Lukassen for excellent technical assistance and to Mr. Jan Prinsen and Mr. Hennie Otten for the histological evaluation.

References

Bowden, G.H. and Hardie, J.M. Microbial variations in approximal dental plaque. Abstracts of the 20th Orca Meeting, Zurich 1973. Helv. Odont. Acta, **34**, 45, (1973).

Hoeven, J.S. van der. A slime producing microorganism selected in dental plaque of rats by glucose feeding. Chemical composition of extracellular slime elaborated by *Actinomyces viscosus*, strain Nyl. Caries Res. **8** 193-210 (1974).

Hoeven, J.S. van der, Mikx, F.H.M., Konig, K.G. and Plasschaert, A.J.M. Plaque formation and dental caries in gnotobiotic and SPF Osborne-Mendel rats, associated with *Actinomyces viscosus*. Caries Res. **8**: 211-233 (1974).

Keyes, P.H. Dental caries in the molar teeth of rats II. J. Dent. Res. **37** 1088-1099 (1958).

Konig, K.G. Dental caries and plaque accumulation in rats treated with stannous fluoride and penicillin. Helv. Odont. Acta **3** 39-44 (1959).

Konig, K.G. Moglichkeiten der Kariesprophylaxe beim Menschen und ihre Untersuchung im kurzfristigen Rattenexperiment (Hans Huber Bern 1966).

Parker, R B and Creamer, H.R. Contribution of plaque polysaccharides to growth of cariogenic microorganisms Arch. oral Biol **16**· 855-862 (1971)

Socransky, S.S. Relationship of bacteria to the etiology of periodontal disease J dent Res **49** 203-222 (1970).

Stoppelaar, J D de, Houte, J van and Backer Dirks, O. The relationship between extracellular polysaccharide-producing streptococci and smooth surface caries in 13-year old children Caries Res. **3** 190-199 (1969)

Theilade, E., Wright, W H , Borglum Jensen, S and Loe, H Experimental gingivitis in man II. J periodontal Res **1** 1-13 (1966).

VI

THE INTERACTION OF *ACTINOMYCES VISCOSUS* WITH ORAL STREPTOCOCCI IN PLAQUE OF GNOTOBIOTIC RATS. ECOLOGICAL SIGNIFICANCE OF ANTIBACTERIAL ACTIVITY IN PLAQUE

J.S. VAN DER HOEVEN, F.H.M. MIKX
A.J.M. PLASSCHAERT AND K.G. KONIG

Institute of Preventive and Community Dentistry

and

J.C MALTHA

Laboratory of Oral Histology
University of Nijmegen

Introduction

The gnotobiotic animal has been used in order to study the effect of a particular microbial interaction in plaque. (Mikx *et al.* 1972). It was found that the presence of *Veillonella* in plaque has a caries reducing effect, possibly due to the utilization of lactic acid, thus suggesting that the variation in the net production of acid was the critical parameter under these conditions.

Since plaque forms a diffusion barrier, the thickness of the layer may be another important parameter that determines the cariogenicity of plaque. Based on this assumption it may be speculated that the introduction of a plaque-forming microorganism will lead to increased cariogenicity. This hypothesis is supported by the previous observation that the establishment of the plaque-forming microorganism *A. viscosus* in SPF rats resulted in an increased cariogenicity while both *A. viscosus* alone and the SPF microflora were found to be a low cariogenic (v.d. Hoeven *et al.* 1974).

The present experiments were aimed at testing the hypothesis outlined above, by associating *A. viscosus* with oral streptococci in gnotobiotic rats. It may be assumed that this experimental model is still too complex since many other interactions between the microorganisms may occur. The ecological significance of microbial antagonism was investigated in further detail.

Methods

Microorganisms

The test organism *A. viscosus* strain Ny1 (v.d. Hoeven 1974) was grown in actinomyces broth (BBL); *Strep. mutans* strain C67-1 (De Stoppelaar *et al.* 1969) *Strep. sanguis* strain Ny101 (Mikx *et al.* 1974) and *Strep. mutans* strain OMZ 176 (Guggenheim 1968) were grown in brain-heart infusion broth (Difco). The inocula (24-h cultures of the strains in 5-ml screw-cap vials) were applied with cotton swabs. If required the inocula of two strains were applied one after the other at the same occasion.

Recovery of strains: The establishment of the microflora and the occurrence of infections was checked weekly, except stated otherwise, by mouth-swabs and sampling of feces as described previously (Mikx *et al.* 1972).

At the termination of the experiment, plaque was sampled from the

lower jaw molars with a suction device (Mikx *et al.* 1974). The microbial content of the cecum was tested in Gram-stained smears.

Treatments

A. Di-association with A. viscosus Ny1 and Strep. sanguis Ny101

Forty germ-free Osborne-Mendel rats originating from 8 litters were weaned at the age of 25 ± 2 days. The litters were distributed at random among 10 treatments, 5 of which belonged to the experiment described here. The animals were fed γ-irradiated diets 516 S (Mikx *et al.* 1972) or 516 G (modification of 516 S, in which sucrose was replaced by glucose). The treatments were as follows:

1. strain Ny101; diet 516 S.
2. strain Ny101 and strain Ny1; diet 516 S.
3. strain Ny1; diet 516 S.
4. strain Ny1; diet 516 G.
5. strain Ny101 and strain Ny1; diet 516 G.

Treatments were carried out in separate isolators. The experimental period was 41 days.

B. Di-association with A. viscosus Ny1 and Strep. mutans C67-1

In the first experiment it was tested whether the combination of strain Ny1 and strain C67-1 could be established in germfree rats. Sixteen OM rats 30 ± 2 days old originating from 4 litters were distributed over 2 treatment groups of 8 rats. The rats were inoculated as follows: treatment 1, strain C67-1 and treatment 2, strain C67-1 and strain Ny1. Each treatment was carried out in a separate isolator containing two cages with 4 rats. All rats were fed diet 540S (Mikx *et al.* 1972). Since the presence of Ny1 in treatment 2 could not be demonstrated on the 5th and 9th day after inoculation, the animals were reinoculated at day 22 by syringe with 0.1 ml of a suspension of Ny1 containing 10^{11} cells/ml. In addition one rat, mono-associated with Ny1, was introduced into one of the two cages of treatment 2. The experimental period was 41 days.

The second experiment was undertaken in order to study the effect of the inoculation sequence on the establishment of Ny1 and C67-1. It was aimed to enhance the chance for the establishment of Ny1 by inoculating this strain several days prior to the inoculation of C67-1. Twenty-eight OM rats 27 ± 2 days old originating from 5 different litters were distributed among 6

treatments. Each treatment was carried out in 2 cages. The effect of the inoculation sequence was studied in rats on a sucrose diet and a glucose diet as well. The arrangement of the treatments and the schedule of inoculations is shown in Table I. On 4 occasions samples were taken from one rat of each cage: mouthswabs were taken at days 3, 10 and 24 after the last inoculation and plaque was sampled at the end of the experiment thus giving a total of 8 bacteriological samples from each treatment group of rats. Upper half jaws from two animals per treatment were processed for electron microscopy of the plaque.

Table I **Experiment 2**
The interaction of *A. viscosus* Ny1 and *Strep mutans* C 67-1.

Arrangement of treatments and schedule of inoculation

Treatment no.	1	2	3	4	5	6
Isolator	I	III	III	II	IV	IV
No. of rats	6	4	4	6	4	4
Diet	540S	540S	540S	540S	540G	540G
Strains inoculated						
on day 1	C 67-1	C 67-1	Ny1	C 67-1	C 67-1	Ny1
on day 2	C 67-1	C 67-1	Ny1	C 67-1	C 67-1	Ny1
on day 3	–	Ny1	C 67-1	–	Ny1	C 67-1
on day 4	–	Ny1	C 67-1	–	Ny1	C 67-1

C. Di-association with A. viscosus Ny1 and Strep. mutans OMZ 176

As the establishment of *A. viscosus* Ny1 in the presence of *Strep. mutans* C67-1 encountered serious difficulties, the interaction of strain Ny1 with another strain of *Strep. mutans*, strain OMZ 176, was additionally tested in a small experiment. Ten young OM rats originating from 4 different litters were available and distributed over two treatments. Both treatments were carried out in the same isolator containing two cages with 5 rats each. All rats were inoculated with strains Ny1 and OMZ 176 and fed diet 540 S or diet 540 F (wheat flour substituted for sucrose). One week after inoculation and at the end of the experiment plaque was collected from one sacrificed rat from each treatment. The experiment was terminated 38 days after inoculation.

Electron Microscopical Procedures

The molars were separated and each was fixed in a formaldehyde-glutaraldehyde fixative for 3 hours at room temperature followed by a second fixation in 1.33% OsO_4 in 0.067 M S-collodine, pH 7.2 at 4°C for 2 hours.

The molars then were dehydrated, embedded in Epon, decalcified in a EDTA-glutaraldehyde mixture at pH 7.2; again fixed in OsO_4, dehydrated and reembedded in Epon, according to Kalberer *et al.* (1971). Ultrathin sections were cut on a Reichert OmU2 ultramicrotome using glass knives.

The sections were stained with uranylacetate and leadcitrate. In some cases polysaccharides were demonstrated using a thiosemicarbazide treatment according to Guggenheim and Schröder (1967). All preparations were examined in a Philips EM200.

Evaluation of caries and plaque

Smooth surface caries, plaque and fissure lesions were scored according to Keyes (1958), König (1959) and König (1966), respectively.

Assay of bacteriocin-like activity

It was observed that C67-1 inhibited the proliferation of Ny1 in plaque. Therefore it was tested whether C67-1 exhibited antibacterial activity in vitro.

Medium. The assays of inhibitory action were performed on a solid medium composed of brain-heart infusion 37 g/l; 0.2% soluble starch and 1.5% agar.

Direct spray method. 24 h. stab cultures in agar plates were covered by spraying a 24 h. culture of the indicator organism on the agar surface. The plate was then reincubated for 24 h. under anaerobic or aerobic conditions. Anaerobiosis was obtained using Brewer jars filled with 10% CO_2 and 90% N_2.

Reverse side spray method. The test organism was inoculated as a shallow stab culture; the reverse side of the agar was used as culture surface for the indicator strain.

Production on semi-solid media. The strains were grown on media containing brain-heart infusion 37 g/l and agar 0.4%. After 24 h. of incubation under aerobic or anaerobic conditions it was attempted to extract the activity by a 'freeze-thaw' technique (Litkenhous and Liu, 1967). The solution obtained was tested on its inhibitory action in the following way: wells with a diameter of 4 mm were punched in the agar and filled with test solution; the indicator strain was subsequently sprayed.

pH measurement

The pH surrounding the stab cultures was measured in the following way pieces of agar around stab cultures of each of the test strains were cut out and the pH of the supernatant fluid which was obtained after freeze-thawing and subsequent centrifugation of the agar, was measured

Assay of hydrogen peroxide

Hydrogen peroxide was determined according to Hoogendoorn and Moorer (1974) A solution (2 5 ml) containing between 10^{-6} and 5 10^{-5}% of H_2O_2 was pipetted into a cuvet A solution (0 5 ml) containing 1 mg horse radish peroxidase and 10 mg *o*-dianisidine HCl in 0 2 M phosphate buffer pH 7 0 was added and mixed Absorbances were measured at 436 nm and the amount of H_2O_2 in the sample was determined by reference to a standard curve Strains were grown in closed vials in brain-heart infusion broth for 24 h at 37° Since H_2O_2 is broken down in brain-heart infusion broth the production of H_2O_2 cannot be measured during growth The cells were centrifuged and washed in 0 85% saline and suspended in 0 1 M phosphate buffer pH 7 supplemented with 1% glucose to give a suspension with an absorbance of 0 5 at 550 nm H_2O_2 production was measured at 30-min intervals during incubation of the cells at 37°

Results

A Diassociation with A viscosus Ny1 and Strep sanguis Ny101

The recovery of the strains and the results of the experimental di-association on the development of plaque and caries are shown in Table II The presence of the inoculated strains could be demonstrated in all treatments during the experimental period The median percentage of strain Ny1 was found to be higher on the glucose diet than on the sucrose diet On both diets the occurrence of strain Ny1 was associated with a high plaque score Di-association with *Strep sanguis* and *A viscosus* resulted in a significantly lower number of fissure lesions compared to rats mono-associated with *Strep sanguis* ($p_t < 0\ 01$, this comparison was only tested for rats on the sucrose diet)

Table II Exp. A. Di-association of *A. viscosus* Ny1 and *Strep mutans* Ny101. Averages ± standard deviations of plaque score, caries scores (dentinal fissure (T) lesions and smooth surface (E) lesions) and median percentages of inoculated strains in gnotobiotic rats associated with *A. viscosus* Ny1 and *Strep sanguis* Ny101 on diet 516S (sucrose) or diet 516 G (glucose).

Treatment	1	2	3	4	5
Strains	Ny1	Ny1+Ny101	Ny101	Ny1	Ny1+Ny101
Diet	516S	516S	516S	516G	516G
Plaque	2.8±0 4	1.9±0.3	0 4±0.5	2.5±0 5	2.5±0.5
Smooth surface lesions	1.5±2 4	0	0	1 0±2.0	0
Fissure lesions	0.1±0 3	0.9±1.0	3.3±1 4	0.8±1.4	0.6±0 9
	n.s.	$p_t < 0.01$		n s	
Number of rats	8	8	8	8	8
Median percentages in plaque					
A. viscosus Ny1		15 (0-90)*			75 (0-80)*
Strep. sanguis Ny101		85 (10-100)*			25 (19-100)*

* range n s. = not significant

B. Di-association with A. viscosus Ny1 and Strep. mutans C67-1

Recovery of strains in experiment 1. The presence of strain C67-1 could be demonstrated in both treatments during the whole experimental period Strain Ny1 could not be demonstrated in the samples taken 5 days after inoculation and therefore the animals were inoculated again with a large quantity of cells applied by syringe. Five days later strain Ny1 was found to occur in a single animal and in this animal the strain was still present at the end of the experiment.

Strain Ny1 also persisted in the newly introduced animal which was mono-inoculated with strain Ny1 and subsequently contaminated with strain C67-1 by its cage mates.

At no occasion strain Ny1 was found in feces or cecum content

Recovery of strains in experiment 2. The presence of strain C67-1 could be demonstrated in all treatments. The recovery of strain Ny1 in the respective treatments is given in Table III, which shows a distinct effect of the inoculation sequence in the glucose-fed animals. When strain Ny1 was first inoculated, its presence was demonstrated in 7 of the total of 8 oral samples that were taken during the experimental period. When the inoculation sequence was reversed, strain Ny1 could only be recovered once. No effect of the inoculation sequence was observed in the sucrose groups. Strain Ny1 could not be detected in Gram-stained smears of the cecum content.

Table III Recovery of *A. viscosus* Ny1 from rats that were inoculated with Ny1 and *Strep. mutans* C 67-1 in different sequences and fed a sucrose or a glucose diet. The number of positive samples from a total of 8 oral samples is given.

Treatment		Recovery of
sequence of inoculation	diet	*A. viscosus*
Ny1; C 67-1	540S	1
Ny1; C 67-1	540G	7
C 67-1; Ny1	540S	1
C 67-1, Ny1	540G	1

Plaque and caries results

The averages of plaque and caries scores in experiments 1 and 2 are summarized in Table IV. In none of the animals massive plaque accumulation was found and no smooth surface caries was scored. The presence of strain Ny1, as revealed by bacteriological methods, had no effect on the individual plaque score.

The numbers of fissure lesions of various severity were not found to be influenced by the inoculation with Ny1. The cariogenicity of strain C67-1 was found to be reproducible (experiments 1 and 2). Strain C67-1 was not significantly more cariogenic on the sucrose diet than on the glucose diet.

Table IV Exp. B. Di-association of *A. viscosus* Ny1 and *Strep. mutans* C67-1. Averages of plaque score and dentinal fissure (T and B) lesions in rats inoculated on different sequences with *A. viscosus* Ny1 and *Strep. mutans* C67-1 and fed on diets 540S (sucrose) or 540 G (glucose).

Experiment	- 1 -		- 2 -					
Treatment	1	2	1	2	3	4	5	6
Strains and sequence of inoculation	C 67-1	C 67-1, Ny1 simulta-neously	C 67-1	C 67-1 Ny1	Ny1 C 67-1	C 67-1	C 67-1 Ny1	Ny1 C 67-1
Diet	540S	540S	540S	540S	540S	540G	540G	540G
Number of rats	8	8	6	4	4	6	4	4
Plaque	1.0±0.5	1.0±0.0	1.0±0.0	0.8±0.5	1.0±0.0	0.8±0.4	1.0±0.0	0.8±0.5
Fissure lesions T	9.6±0.7	9.9±1.8	11.5±0.9	11.0±1.1	11.0±0.8	10.7±1.1	11.0±1.1	11.0±0.8
B	7.4±1.7	8.0±1.8	9.3±2.0	7.2±2.4	8.5±1.3	7.5±3.2	8.7±1.0	8.5±1.9
	n.s.		n.s.					

n.s. = not significant

Electron microscopical findings

The presence of *Strep. mutans* C67-1 and *A. viscosus* Ny1 in plaque was checked electron microscopically. All findings refer to fissure plaque since smooth surface plaque was lost in most cases when the samples were re-embedded.

Mono-associated animals with C67-1. A striking difference was observed in the distribution of the bacterial cells between the glucose and the sucrose plaque. The sucrose plaque was built up of loosely packed cells most of which were apparently intact and the appearance of the plaque was similar all through the fissure (Figure 1). The cells in the glucose plaque were densely packed and areas with either large or small numbers of degenerating cells were seen alternating throughout the fissure and apparently without preferential sites. Fig. 2 shows plaque from an area near the enamel surface on the bottom of the fissure and Fig. 3 shows an area at the outside of the plaque.

Di-associated animals. No electron microscopical evidence was obtained for the presence of strain Ny1 in any of the treatment groups.

In both the sucrose and the glucose groups (figs. 4 and 5) only streptococcal cells could be identified with certainty; all deviating morphological types were also found to occur in the rats that were mono-associated with C67-1 and therefore represented different states of degeneration of the same microorganism (compare figures 3 and 4).

C. Di-association with A. viscosus Ny1 and Strep. mutans OMZ 176

Strain Ny1 was found to be quite well established in the presence of strain OMZ 176. Considerable plaque formation was found in both dietary groups of rats.

Assay of antibacterial activity

It was found that strain C67-1 produces inhibition zones in plate assays against strain Ny1. No inhibitory action of strains OMZ 176 or Ny101 against strain Ny1 was observed. It was noted that the inhibition zones produced by C67-1 varies in size in different tests. The inhibition zones observed were not due to acid production by the stab cultures, for the pH surrounding the stab cultures was about equal for all 3 the test strains: 6.4, 6.3 and 6.4 for strains C67-1, OMZ 176 and Ny101 respectively, and these values are tolerated by strain Ny1. Attempts to isolate antibacterial activity produced by strain C67-1 failed. When strain C67-1 was cultured on a semi solid brain-heart infusion medium, no antibacterial activity of the supernatant-fluid which was

obtained after freeze-thawing and centrifugation of the medium, could be demonstrated.
No activity could be demonstrated in broth cultures of C67-1.

Production of hydrogen peroxide. All three strains of streptococci were found to produce hydrogen peroxide. A linear increase of the H_2O_2 concentration was observed for at least 1 h. of incubation. The amount of H_2O_2 produced per hour by cell suspensions of equal density of the test strains was $2.8.10^{-5}$% $3.1.10^{-5}$% and $3.4.10^{-5}$% H_2O_2 for strains C67-1, OMZ 176 and Ny101 respectively.

Discussion

According to the hypothesis tested in the present experiment, the interaction of the plaque-producing microorganism *A. viscosus* with oral streptococci was expected to result in highly cariogenic plaque. The interaction with *Strep. sanguis* Ny101 resulted in the formation of large amounts of plaque harbouring both microorganisms, but contrary to the hypothesis the plaque was not significantly more cariogenic than the plaque formed by *A. viscosus* alone and the cariogenicity was even found to be reduced compared to the plaque built up by *Strep. sanguis* alone. These findings suggested that the caries-promoting effect of increased thickness was completely abolished by the decreased proportion of *Strep. sanguis* in plaque.

A powerful inhibitory effect of *Strep. mutans* C67-1 on *A. viscosus* was observed. It is noted that this inhibitory action was unique for *Strep. mutans* strain C67-1 and not for the species, as was demonstrated by the symbiosis of Ny1 with *Strep. mutans* OMZ 176. This was in accordance with the findings reported by Holmberg and Hallander (1971) who did not observe antagonistic effects between *Strep. mutans* NCTC 10449 and strains of *A. viscosus*. Recently Holmberg and Hallander (1973) showed that the antibacterial effect of *Strep. sanguis* strains was due to the production of hydrogen peroxide. The findings that each of the 3 test strains used in the present experiment produce H_2O_2 at an equal rate, makes it very unlikely that the antibacterial effect of C67-1 was due to H_2O_2 production. The failure to demonstrate the presence

of Ny1 by bacteriological techniques, except in one group, might due to the fact that C67-1 inhibited the growth of Ny1 on agar media. However, the bacteriological results are in accordance with the electron microscopical observation which failed to detect Ny1 in the fissure plaque.

In addition, no increased plaque formation was found in the animals inoculated with C67-1 and Ny1 thus suggesting that Ny1 was absent or at least metabolically inactive while in all previous tests the establishment of Ny1 was associated with a high plaque score.

That Ny1 was recovered in considerable numbers from the glucose-fed animals where it was inoculated first, but could not be demonstrated in the electron micrographs might indicate that it originated in fact from other sites than the fissures. The alternative explanation, that Ny1 was present in the fissures but could not be distinguished from C67-1 electron microscopically seems unlikely. However no definite answer can be given since electron micrographs of Ny1 plaque are not yet available.

The inhibitory substance produced by C67-1 was diffusable and the failure of the attempt to produce a cell-free preparation of the substance suggested that it was labile. The activity in vitro was directed against several other Gram-positive species and in addition it was found that C67-1 inhibited the establishment of *Bacterionema matruchotii* (strain ATCC 14266), but not of the Gram-negative species *Veillonella alcalescens* (strain OMZ 193) in the plaque of gnotobiotic rats (unpublished results).

The production of bacteriocin-like substances was reported for various types of streptococci originating from dental plaque (Kelstrup and Gibbons, 1969; Kelstrup *et al.* 1971) or the throat (Piatkowsky and Szropinska, 1971) and group A streptococci (Tagg *et al.* 1971).

It was suggested that the present observation in gnotobiotic animals reflects the action of a bacteriocin or rather a streptococcin (Piatkowsky and Szropinska, 1971) from C67-1.

In how far the findings from this model could be extrapolated to the microbiologically complex situation in dental plaque is hard to speculate. As suggested by Kelstrup and Gibbons (1969), it might well be that bacteriocins are rapidly inactivated when exposed to the various enzymes in dental plaque. Some evidence for the ecological significance of streptococcins in the microbiota of the respiratory tract was presented by the experiments of Sprunt and Redman (1969) and the findings of Piatkoswky and Szropinska (1971).

Finally, it was demonstrated that the cariogenicity of *Strep. mutans*

C67-1 was very similar on glucose and sucrose diets. That the cariogenicity of polysaccharide producing streptococci in mono-associated rats is not necessarily higher on sucrose than on glucose was previously suggested by the experiments of Rosen (1969).

In our opinion polysaccharide synthesis is a caries promoting factor and the present results suggest that the absence of polysaccharide synthesis was compensated by the higher density of bacterial cells resulting in a higher metabolic activity in the glucose plaque.

References

Guggenheim, B.: Streptococci of dental plaques. Caries Res. 2: 147-163 (1968).

Guggenheim, B. and Schroeder, H.E.: Biochemical and morphological Aspects of Extracellular Polysaccharides produced by Cariogenic Streptococci. Helv. Odont. Acta **11**: 131-152 (1967).

Hoeven, J.S. van der: A slime producing microorganism in dental plaque of rats, selected by glucose feeding. Caries Res. **8**: 193-210 (1974).

Hoeven, J.S. van der; Mikx, F.H.M.; Kónig, K.G., Plasschaert, A.J.M.: :Plaque formation and dental caries in gnotobiotic and SPF-Osborne Mendel rats associated with *Actinomyces viscosus* Ny1. Caries Res. 8: 211-223 (1974).

Holmberg, K. and Hallander, H.O.: Interference between Gram positive microorganisms in dental plaque. J. dent. Res. **51**: 588-595 (1971).

Holmberg, K. and Hallander, H.O.: Production of bactericidal concentrations of hydrogen peroxide by *Streptococcus sanguis*. Arch. oral Biol. **18**: 423-434 (1973).

Hoogendoorn, H. and Moorer, W.R.: Lactoperoxidase in the prevention of plaque accumulation, gingivitis and dental caries (I). Odont. Revy **24**: 355-366 (1973).

Kalberer, P.U.; Schroeder, H.E.; Guggenheim, B. and Mühlemann, H.R.: The microbial colonization in fissures. A morphological and morphometric study in rat molars. Helv. odont. Acta **15**: 1-14 (1971).

Kelstrup, J. and Gibbons, R.J.: Inactivation of bacteriocins in the intestinal canal and oral cavity. J. Bact. **99**: 888-890 (1969).

Kelstrup, J. and Gibbons, R.J.: Bacteriocins from human and rodent streptococci. Arch. oral Biol. **14**: 251-258 (1969).

Kelstrup, J.; Richmond, S.; West, C. and Gibbons, R.J.: Fingerprinting human oral streptococci by bacteriocin production and sensitivity. Arch. oral Biol. **15**: 1109-1116 (1970).

Keyes, P.H.: Dental caries in the molar teeth of rats. II. J. dent. Res. **37**: 1088-1099 (1958).

König, K.G.: Dental caries and plaque accumulation in rats treated with stannous fluoride and penicillin. Helv. odont. Acta **3**: 39-44 (1959).

König, K.G.: Möglichkeiten der Kariesprophylaxe beim Menschen und ihre Untersuchung im kurzfristigen Rattenexperiment. (Haus Huber, Bern 1966).

Litkenhous, C. and Liu, P.V.: Bacteriocin produced by *Bordetella pertussis*. J. Bact. **93**: 1484-1488 (1967).

Mikx, F.H.M.; Hoeven, J.S. van der; König, K.G.; Plasschaert, A.J.M. and Guggenheim, B.: Establishment of defined microbial ecosystems in germ-free rats. Caries. Res. **6**: 211-223 (1972).

Mikx, F.H.M.; van der Hoeven, J.S.; Plasschaert, A.J.M. and König, K.G.: Effect of *Actinomyces viscosus* on the establishment and symbiosis of *Streptococcus mutans* and *Streptococcus sanguis* in SPF rats on different sucrose diets. Caries Res. **8**: (1974).

Piatkowsky, K. and Szropinska, D.: Studies on bacteriocinogeny of streptococcus strains. Arch. Immunol. Therap. Exp. **19**: 137-145 (1971).

Rosen, S.: Comparison of sucrose and glucose in the causation of dental caries in gnotobiotic rats. Arch. oral Biol. **14**: 445-450 (1969).

Sprunt, K. and Redman, W.: Evidence suggesting importance of role of interbacterial inhibition in maintaining balance of normal flora. Ann. internal Medicine: **68**: 579-590 (1968).

Stoppelaar, J.D. de; van Houte, J. and Backer Dirks, O.: The relationship between extracellular polysaccharide producing streptococci and smooth surface caries in 13-year-old children. Caries Res. **3**: 190-199 (1969).

Tagg, J.R., Read, R.S.D. and Mc Given, A.R.: Bacteriocine production by group A streptococci. Pathology **3**: 277-278 (1971).

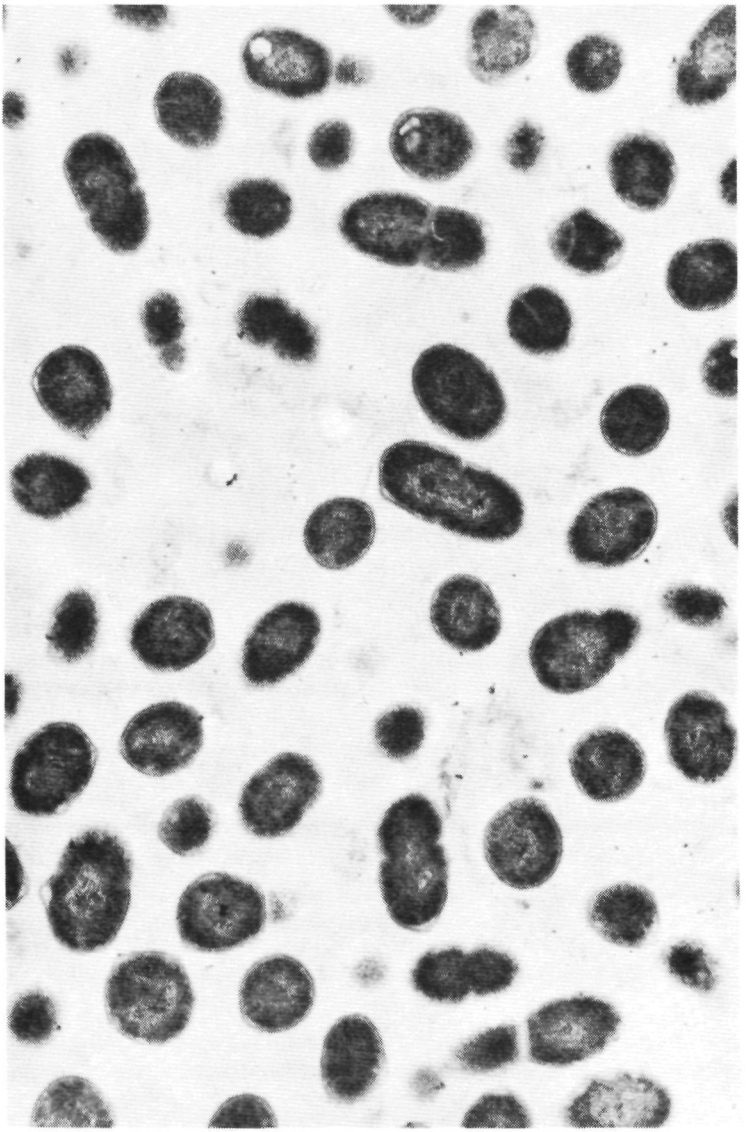


Fig. 1 Section of fissure plaque in the deepest part of a fissure. Cells of *Strep. mutans* embedded in polysaccharide. The rat was mono-associated with *Strep. mutans* C67-1 and fed sucrose diet 540 S. x 12,000.

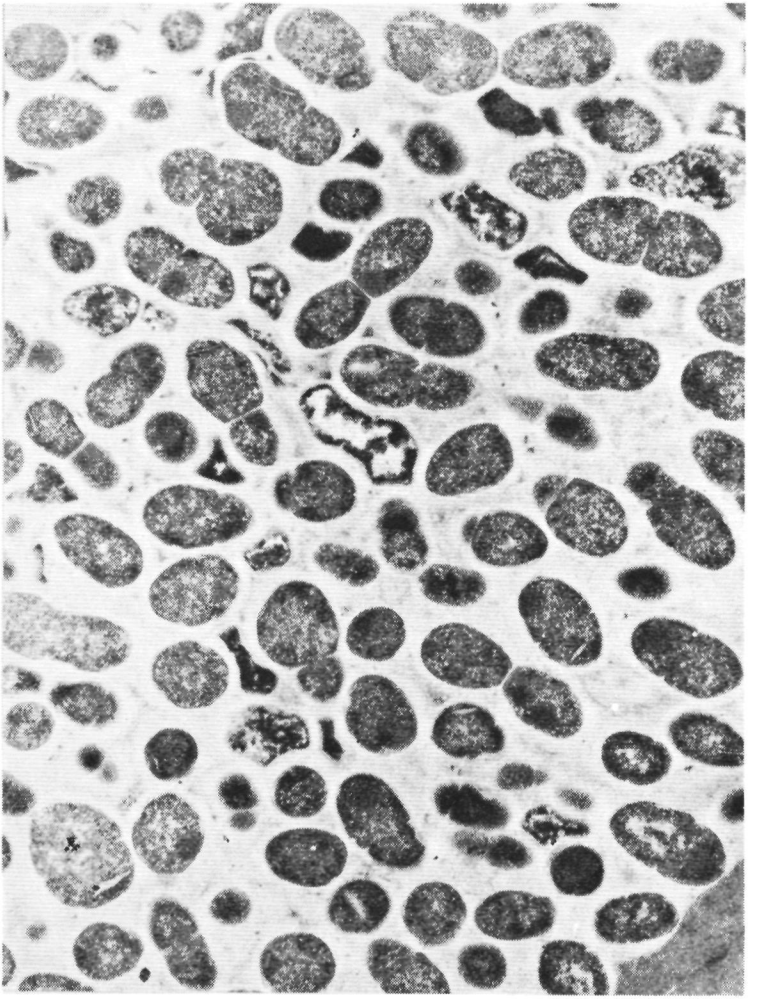


Fig. 2 Section of fissure plaque in the deepest part of a fissure showing densely packed streptococcal cells most of which looking intact. The rat was mono-associated with *Strep. mutans* C67-1 and fed glucose diet 540 G. x 12,000.

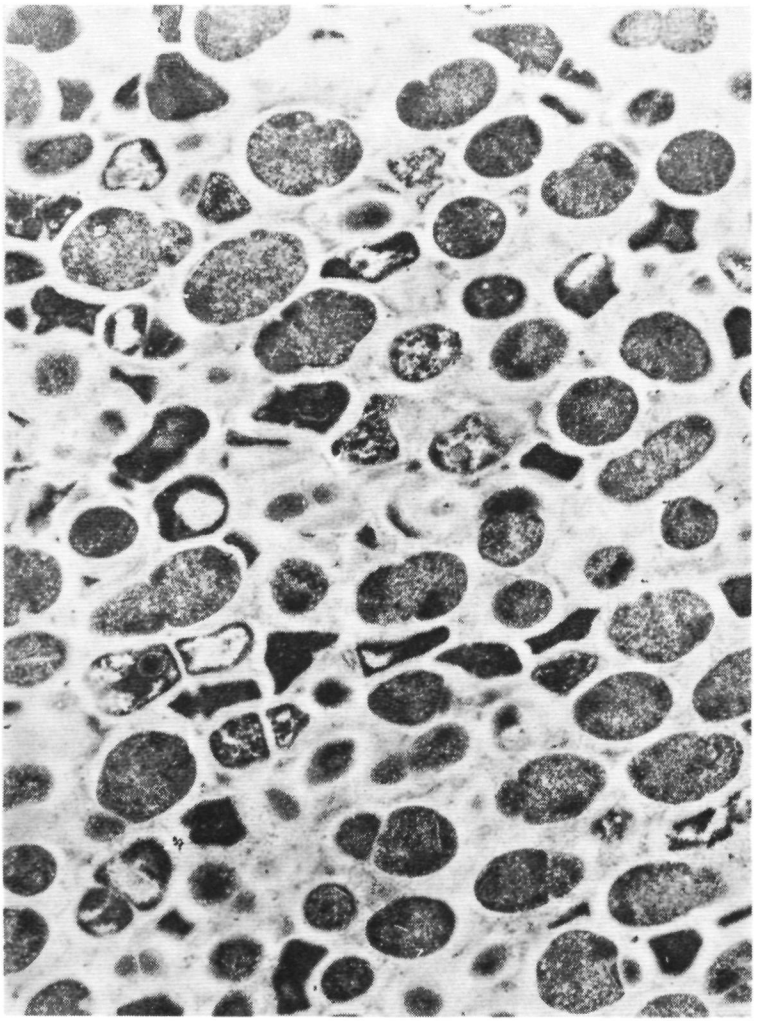


Fig. 3 Section of fissure plaque at the outside of the same fissure as in Fig. 2, showing cells of deviating morphology. The rat was mono-associated with *Strep. mutans* C67-1 and fed glucose diet 540 G. x 12,000.

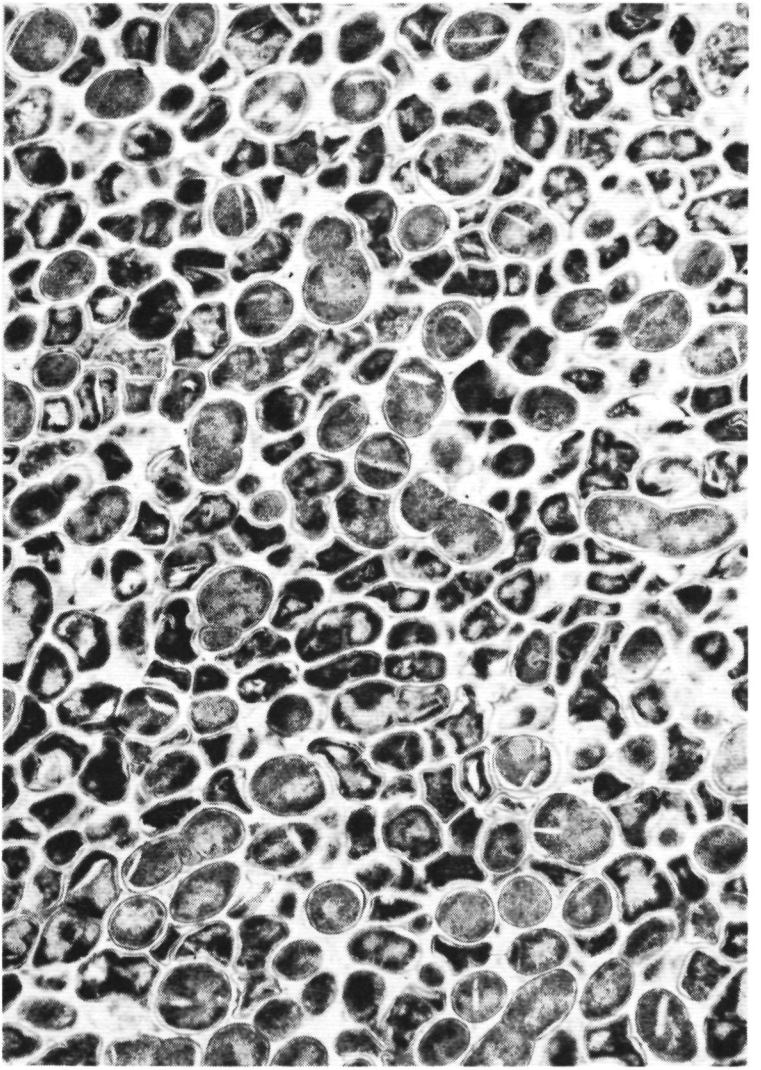


Fig. 4 Fissure plaque from a rat inoculated with *Strep. mutans* C67-1 and *A. viscosus* Ny1 and fed glucose diet 540 G. Different morphological types can be observed at various state of degeneration. x 10,000.

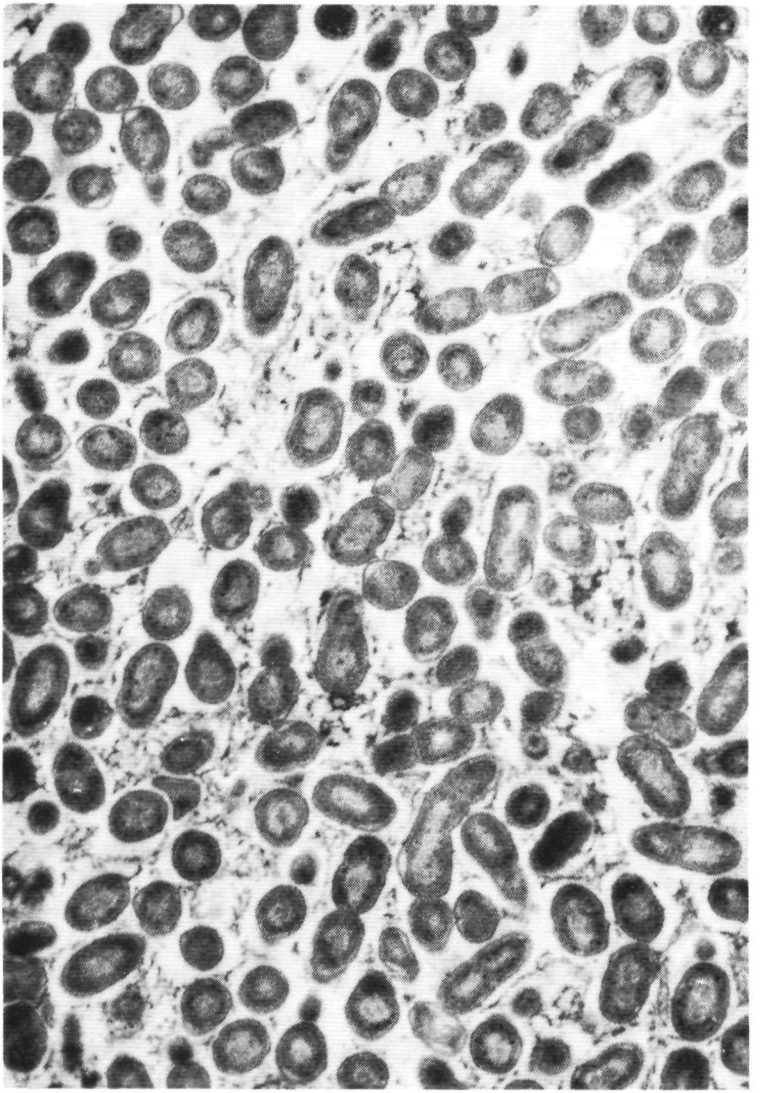


Fig. 5 Fissure plaque from a rat inoculated with *Strep. mutans,* C67-1 and *A. viscosus* Ny1 and fed glucose diet 540 S. The section shows an area near the enamel were streptococcal cells embedded in polysaccharide are seen, while the absence of Ny1 is suggested by the absence of deviating types. Polysaccharide staining x 12,000.

VII

A LEVANSUCRASE FROM *ACTINOMYCES VISCOSUS**

J.S. VAN DER HOEVEN

Institute of Preventive and Community Dentistry
University of Nijmegen

G D. VOGELS AND M F.J. BEKKERS

Department of Microbiology
University of Nijmegen

* Presented for publication in Caries Research

Abstract. The present study deals with the production and properties of a levansucrase from *Actinomyces viscosus* which was found to be an extracellular enzyme.

In batch and continuous cultures enzyme activity was low at high external glucose concentration. This relationship may be indirect and result from secondary effects such as the production of inhibitory substances or proteolytic enzymes and in the case of batch cultures by retardation of the growth.

Metal ions seem to be not essential for the activity of levansucrase from *A. viscosus*.

Introduction

The occurrence of levan in human dental plaque after intake of sucrose (Mc Dougall, 1964; Manly *et al.* 1966) has been ascribed to a few oral species: *Streptococcus salivarius* (Wood, 1964), *Streptococcus mutans* (Wood and Critchley, 1966) and *Actinomyces viscosus* (Llory and Frank, 1970). The presence of *A. viscosus* in human dental plaque was demonstrated by Snyder *et al.* (1967) and Gerencser and Slack (1969). Levan is considered to be an extracellular storage carbohydrate. It is degraded by plaque samples (Manly and Richardson, 1968) and various organisms present in the plaque exhibit levanase activity (Da Costa and Gibbons, 1968; van Houte and Jansen, 1968; Mesner, 1970). These findings explain the virtual absence of levan in pooled dental plaque (Hotz *et al.* 1972).

Levansucrase (β-2,6 fructan: D glucose 6-fructosyltransferase EC 2.4.1.10) is produced by many bacteria and the enzymes from *Aerobacter levanicum*, *Bacillus subtilus* and *Strep. mutans* were subject to extensive studies (Ebert and Schenk, 1968, Rapoport, 1966; Carlsson, 1970) and found to be very similar.

The present study deals with the production and properties of a levansucrase from *A. viscosus*, which is an extracellular enzyme.

Methods

Microorganisms. The enzyme studied was produced by *Actinomyces viscosus* strain Ny1. The strain has been isolated from dental plaque of rats and its characteristics have been described previously (v.d. Hoeven 1974). *A. vis-*

cosus strain Ny1 was shown to induce gross plaque formation in rats, fed on sucrose or glucose diets

Media and growth conditions

A viscosus Ny1 was grown continuously in the following medium Casein hydrolyzate (dialyzed) 2 g, $(NH_4)_2SO_4$ 1,3 g, glucose 10 g or 1 g, Inositol 2 mg, Folic acid 0 005 mg, Biotin 0 005 mg, p-Aminobenzoic acid 0 01 mg, Thiamine HCl 0 5 mg, Riboflavine 0 5 mg, Pyridoxine 12 mg, Calcium pantothenate 1 2 mg, Nicotinic acid 2 5 mg, L-Cysteine HCl 50 mg, $MgSO_4$ $7H_2O$ 0 2 g, $FeSO_4$ $7H_2O$ 10 mg, $MnSO_4$ 10 mg, NaCl 10 mg, per liter 0 1 M-potassium phosphate buffer, pH 7 0

The same medium was used for batch cultures containing various amounts of glucose The ingredients except casein hydrolyzate and phosphate buffer were sterilized in ten-fold concentration by filtration (0 7 μ membrane filter, Sartorius SM16228) Casein hydrolyzate and phosphate buffer were sterlilized at 121°C, for 20 min

The effect of substrates other than glucose on the production of enzyme was tested in batch cultures containing 1% of the respective substrates, pepton (Difco) 10 g/l, and yeast extract (Difco) 5 g/l The media were sterilized at 121°C for 20 min

Continuous growth was performed at 37°C in 250 ml working volume with stirring (600 rpm) under a constant flow of 95% N_2 and 5% CO_2 The pH of the culture was kept constant at 7 0 by the addition of 2N KOH, pH, temperature and KOH consumption were recorded Batch cultures in 250-ml vessels were incubated at 37°C in a shaking water bath, or in a New Brunswick G 24 incubator shaker in an atmosphere of 95% N_2 and 5% CO_2

Bacterial dry weight was determined as follows bacteria in 50 ml culture fluid were harvested by centrifugation, washed with 10 ml water and dried for 2 days at 110°C in pre-weighed containers

Enzyme assay

Levansucrase activity was estimated by measuring the release of reducing sugars or glucose in a standard reaction mixture at 37°C containing 0 125 M sucrose, 0 05 M sodium phosphate buffer or 0 025 M Tris HCl buffer, pH 6 8, and the enzyme The reaction started on the addition of enzyme was stopped by heating the incubation mixture in a boiling water bath for 10 min or by the addition of an equal volume of 0 08 N NaOH The sugars were assayed immediately

One unit of enzyme activity was defined as the amount which liberates 1 μmol of glucose per min. under the above conditions. If glucose was present in the culture it was removed by dialysis against cold phosphate buffer prior to the enzyme assay.

Purification procedures

The enzyme was isolated from a 12 l culture of *A. viscosus* Ny1 grown in dialyzed actinomyces broth (BBL) at constant pH 6.7 under 95% N_2 and 5% CO_2 for 34 h at 37°C.

The cells were removed by centrifugation (30 min; 10.000 g). The culture supernatant was dialyzed for 18 h against cold tap water in order to lower the ionic strength. To absorb the enzyme, 200 ml preswollen DEAE cellulose (Whatman DE 52) were added to 12 l of the dialyzed supernatant and the suspension was stirred for 2 h at 4°C. After sedimentation overnight the supernatant was removed and the slurry was washed 3 times with 0.025 M Tris·HCl buffer, pH 8.0. The DEAE cellulose was then packed in a column and eluted with a gradient of 0 to 0.25 M sodium chloride in the same buffer. The pooled enzyme fractions were dialyzed against 2M urea with 1% (w/v)glycine. Iso-electric focusing (IEF) was performed in a 220 ml column (LKB Uniphor) with a density gradient of 0 to 50% (v/v) glycerol and a pH gradient provided by Ampholine (1% v/v) from 3-10 in 2M urea with 1% w/v glycine. The column was loaded with 85 mg protein. The enzyme was focused for 3 days.

Ampholytes and other low molecular weight substances were removed by passage through a column packed with Sephadex G50 (Vesterberg, 1969).

The purity of the enzyme preparation obtained was tested by polyacrylamide gel electrophoresis.

Electrophoresis was performed on 7% acrylamide gels in Tris·HCl buffer pH 8.9 according to the method described by Maurer (1968) (gelsystem Nr. 1).

Samples were run in triplicate; one column was stained with amido black, 1%(w/v) in 7% (v/v) acetic acid to disclose protein. The second column was incubated in 125 mM sucrose and 0.025 M Tris·HCl, pH 6.8, for 16 h at 37°C to reveal polysaccharide formation in the gel and the third column was incubated for 15 min. in the same mixture. Enzymatic activity, i.e. release of reducing sugars in the gel was then demonstrated according to Gabriel and Shu Fong Wang (1969).

The cells obtained from the 12-l culture were washed once with 0.05 potassium phosphate buffer, pH 6.8, and suspended in 250 ml of this buffer.

To release the enzyme from the cells, the cooled suspension was subjected 5 times for 1 min. to ultrasonification with a Branson sonifier (130 W output).

Analytical methods

Glucose was determined with glucose oxidase-peroxidase (PGO, Sigma). Invertase activity in the PGO enzyme preparation was inhibited with $AgNO_3$ or Tris. The enzymes were then dissolved in 10^{-4} M $AgNO_3$ or 0.5 M Tris. HCl, pH 7.0, respectively. Alternatively glucose was determined with hexokinase and glucose-6 phosphate dehydrogenase.

Fructose was determined with the same method after conversion of fructose-6 phosphate with phosphoglucoisomerase.

The reagents for these determinations were purchased from Boehringer and the assays were carried out according to the prescriptions of the manufacturer.

Reducing sugars were determined by the method of Somogyi (1945). *Ketohexose* was determined according to Dische and Devi (1960). *Carbohydrate* was determined with the phenol-sulfuric acid method (Dubois *et al.*, 1956).

Polysaccharide. Two volumes of absolute ethanol were added to the culture supernatant fluid. The precipitated material was collected by centrifugation, dialyzed against distilled water and lyophilized.

Analysis of polysaccharide. Samples (10 mg/ml) of the polysaccharide were hydrolyzed in 1% oxalic acid for 1 h at 100°C or in 2 N HCl for 4 h at 100°C. Glucose and fructose were assayed in the hydrolyzates.

The hydrolyzates were deionized with a mixed-bed resin (Amberlite MB3) in the carbonate form and concentrated in a vacuum rotating evaporator at 40°.

Separation of the products was performed on Whatman No. 3 MM paper in ethyl acetate-pyridine-water (10:4:3, by vol.) by descending chromatography. The papers were dipped in silver nitrate-sodium hydroxide (Trevelyan *et al.*, 1950). *Protein* was determined according to Hartree's modification (1972) of the Lowry method.

Levanase activity. The hydrolytic activity against the polysaccharide product was tested in an incubation mixture containing 0.025 M Tris·HCl, pH 6.8, polysaccharide (5 mg/ml) and enzyme (0.5 U levansucrase per ml). The amount of fructose released was determined after 24 h of incubation.

Production of levansucrase in continuous cultures

A. viscosus Ny1 was cultivated continuously in casein hydrolyzate medium containing 0.1 or 1.0% of glucose. The data presented in Fig. 1 indicate that the maximum growth rate in 0.1% glucose medium is close to 0.2 h^{-1} and that glucose was the limiting substrate in this medium. The bacterial density and the levansucrase activity were found to be fairly constant with increasing dilution rates up to 0.11 h^{-1}. In a medium containing 1% glucose, this compound is the limiting substrate at dilution rates below 0.04 h^{-1} (fig. 2).

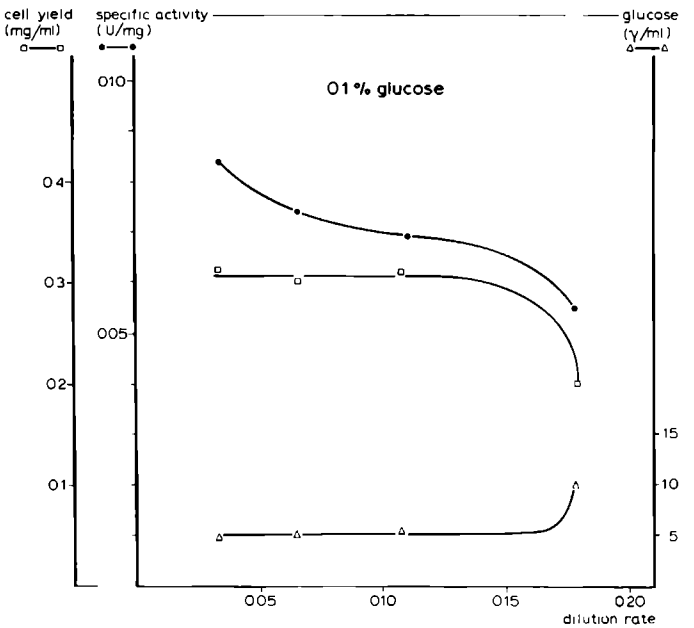


Fig. 1 Production of levansucrase by *A. viscosus* Ny1 in continuous cultures in casein hydrolyzate medium containing 0.1% glucose.

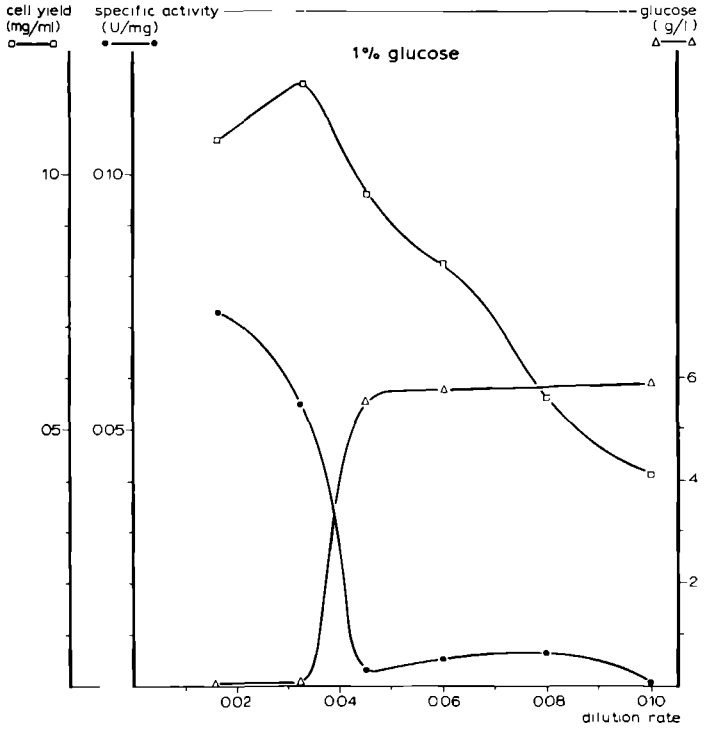


Fig. 2 Production of levansucrase by *A. viscosus* Ny1 in continuous cultures in casein hydrolyzate medium containing 1% glucose.

The rise of the glucose level in the medium at higher dilution rates was accompanied with a decrease of the production of levansucrase.

The bacterial density was found to decrease considerably at increasing dilution rates.

Production of levansucrase in batch cultures. The levansucrase production during growth in casein hydrolyzate medium containing 0.05% glucose is shown in fig. 3. Levansucrase is present in the culture during the logarithmic phase of growth but disappears almost completely when the culture reaches

the stationary phase. The levansucrase production per amount of cells (relative activity = U/ml. A 550 nm) was found to be constant during the logarithmic phase. The disappearance of enzymatic activity in the stationary growth phase is due to the production of an inhibitor or a proteolytic enzyme, since the activity disappears when a mixture of cultures in the logarithmic and the stationary phase is incubated for 30 min. at 37°C.

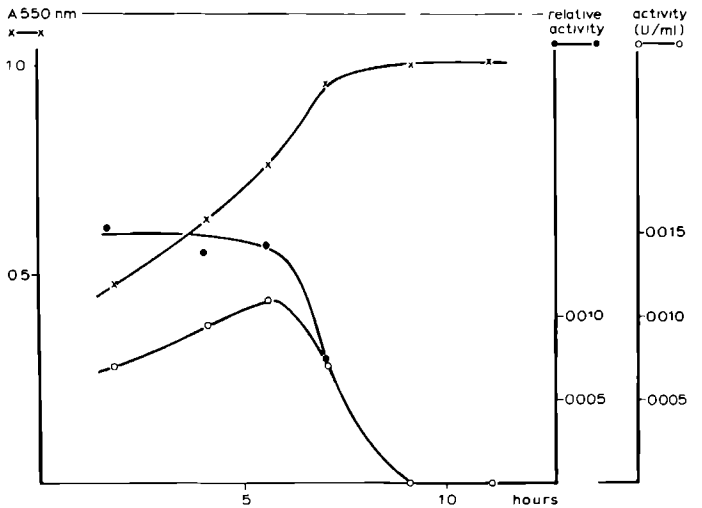


Fig. 3 Production of levansucrase in batch culture in casein hydrolyzate medium containing 0.05% glucose.

Similar results were obtained in media containing higher glucose concentrations. Fig. 4 shows the maximum activities and the optical densities reached in the stationary growth phase that were observed in cultures at various glucose concentrations. The highest activity was observed in a culture containing 1% glucose. The production of enzyme per amount of cells was constant in media containing 0.05% to 1% glucose but was substantially lower in a medium with 1.5% glucose.

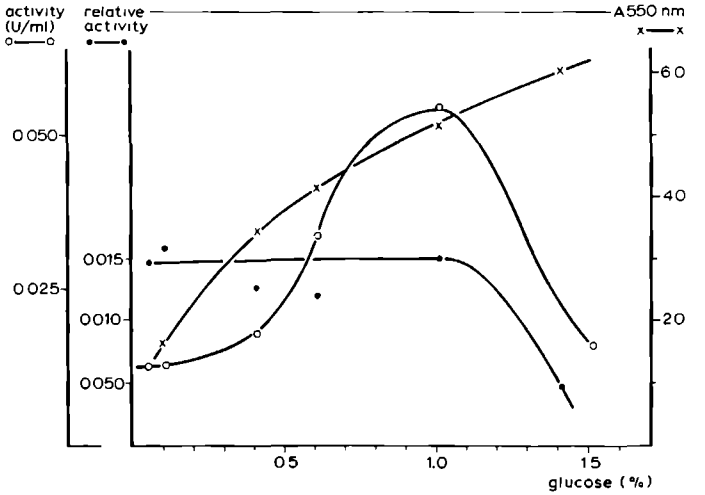


Fig. 4 Maximal activity of levansucrase in batch cultures in casein hydrolyzate medium containing various glucose concentrations. The cell density in the stationary phase of growth is also indicated.

Effect of other substrates on levansucrase production. Table I shows the maximum levansucrase activity observed in cultures grown in pepton-yeast extract medium containing 1% of various substrates. The enzyme is produced also when glucose is replaced by sucrose, pyruvate, m-inositol or glutamate.

Purification

The procedure is given under Methods. A representative elution pattern of the enzyme from DEAE-cellulose is shown in fig. 5. The enzyme was eluted at low ionic strength. The pattern obtained on iso-electric focusing indicates a low iso-electric point of the enzyme since it was focused in the pH region below pH 4.0 (fig. 6). The whole purification procedure is summarized in Table II. The yield of enzyme was 18% and a 165-fold increase in the specific activity was obtained. A repetition of the iso-electric focusing procedure in a shallower pH gradient did not improve the results (fig. 7).

A small proportion (approximately 8%) of the enzyme activity of the culture was found to occur associated with cells. Prolonged sonification of the cells did not result in the release of more levansucrase activity.

Table I Maximal levansucrase activity in cultures of *A viscosus* grown in peptone-yeast extract medium containing 1% of various substrates

substrate	activity U/ml	bacterial density* A 550 nm	Relative activity U/ml A550 nm (10^{-2})
glucose	0.022	1.18	1.9
sucrose	0.028	3.30	0.9
pyruvate	0.005	0.490	1.1
m-inositol	0.027	4.75	0.6
glutamaat	0.012	0.520	2.3

* A 550 nm at maximal levansucrase activity of the culture

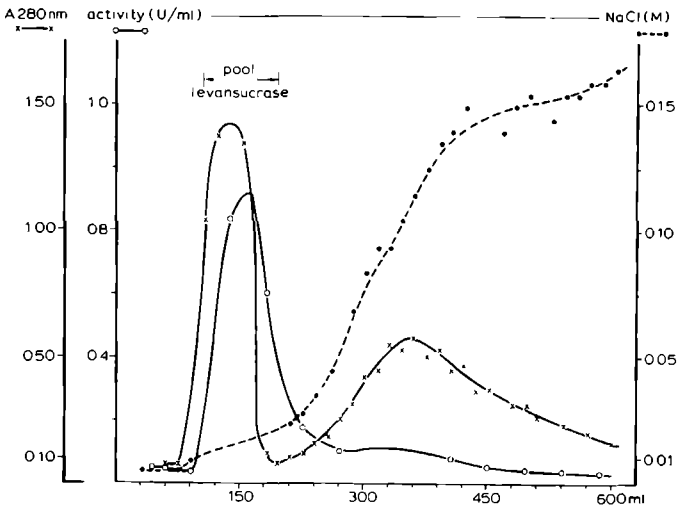


Fig 5 Elution pattern of levansucrase from DEAE-cellulose

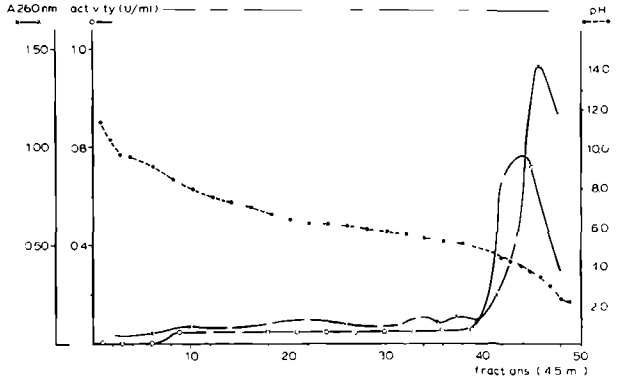


Fig. 6 Elution diagram after iso-electric focusing of levansucrase obtained as the eluate from DEAE-cellulose

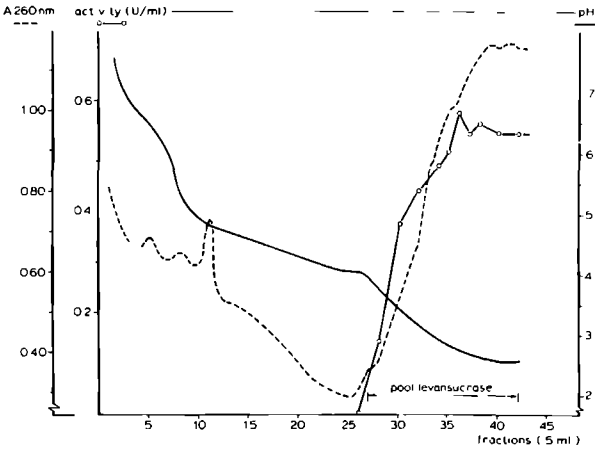


Fig. 7 Elution diagram after repeated iso-electric focusing of levansucrase obtained from the experiment described in Fig. 6.

Table II Purification of levansucrase from *A viscosus*

Step.	volume ml	enzyme conc 10^{-2}.U/ml	Total units U	Protein mg/ml	Specific Activity 10^{-2}.U/mg protein	Yield %	Purification times
Culture supernatant	12.000	3.91	469	4.40	0.89	100	1
Dialyzed culture supernatant	12.000	3.16	379	1.50	2.1	81	2.4
DEAE chromatography and concentration with Aquacide*	16	1167	184	27.25	42.8	40	48
Isoelectric focusing (fig 6)	35	238.8	84	1.63	146	18	165

* Aquacide II (Calbiochem.)

Polyacrylamide gel electrophoresis of the purified enzyme obtained according to the procedure summarized in Table II revealed the presence of several protein bands only one of which exhibited enzymatic activity Both techniques for demonstrating enzyme activity were sensitive, in that activity was detectable even when no protein staining band was observed

Products of the levansucrase reaction The main reaction products from sucrose were glucose, fructose and polysaccharide The ratio of the products determined in a 24 h standard incubation mixture was found to be glucose fructose polysaccharide = 1 0 38 0 65 The presence of these compounds together with sucrose was also obvious in paper chromatograms made with the reaction mixture Moreover a very faint spot with a mobility lower than sucrose and most likely an oligosaccharide, was observed when large quantities of the reaction mixture were applied The oxalic acid hydrolyzate of the polysaccharide revealed only one spot identical to fructose Hydrolysis in 2 N HCl resulted in complete loss of fructose and no glucose could be detected in the hydrolyzate These results indicate that the polysaccharide is a polymer of fructose Moreover an enzymatic assay of glucose in the hydrolyzate confirmed the absence of this compound The ketohexose content (Dische and Devi, 1960) of the polysaccharide was found to be equal to the carbohydrate content determined with phenol-sulfuric acid using fructose as a standard

Kinetics of the reaction The release of glucose or reducing sugar was linear with the time up to 2 h of incubation On incubation of the polysaccharide with the enzyme, no fructose was found to be released which indicates the absence of levanase activity

Specificity Sucrose and raffinose could serve as substrates for polysaccharide (fructan) synthesis, while glucose, fructose, maltose or lactose could not

Effect of the temperature The enzyme exhibited maximal activity at 45°C, a rapid decline of the activity was observed at higher temperatures (fig 8) The effect of storage of the enzyme for 15 min at various temperatures is shown in fig 9 At 4°C the concentrated enzyme (2 4 U/ml) could be stored for 3 months in 0 025 M Tris HCl, pH 6 8, without loss of activity

Effect of pH and buffer The optimum pH was 6 8 and 50% activity was exhibited at pH 5 3 and 9 1, respectively (fig 10)

At pH 6 8 the enzyme was found to be less (-10%) active in 0 05 M citrate buffer and more (+25%) active in 0 025 M Tris buffer than in 0 05 M phosphate buffer

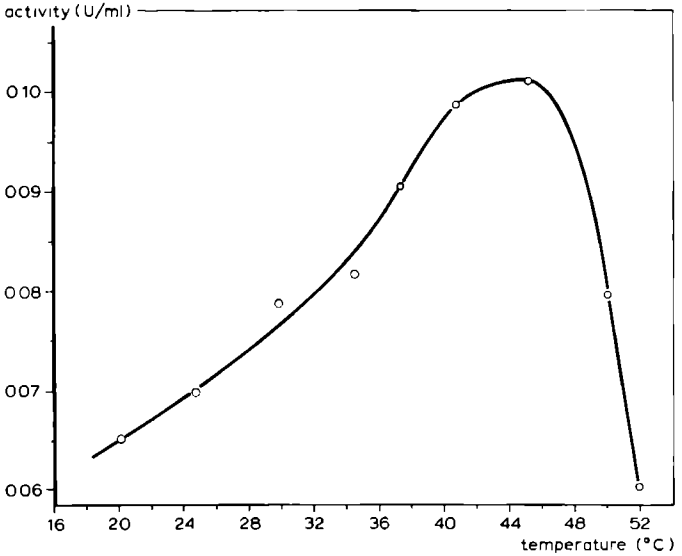


Fig. 8 Effect of the temperature on the activity of levansucrase. The activity was determined by measuring the release of reducing sugars in the standard mixture for 30 min.

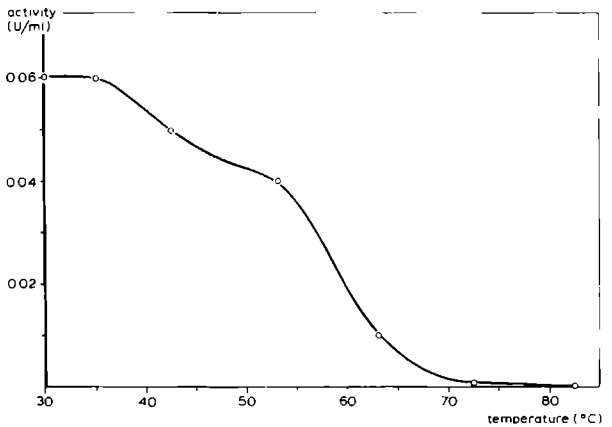


Fig. 9 Effect of storage for 15 min. at various temperatures on the activity of levansucrase. The activity was determined by measuring the release of reducing sugars in the standard reaction mixture after incubation for 30 min. at 37°C.

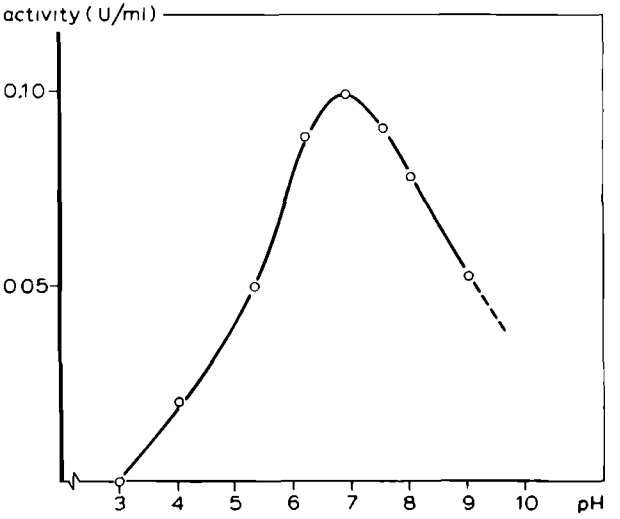


Fig 10 Effect of pH on the activity of levansucrase. The activity was determined by measuring the release of reducing sugars in the standard reaction mixture after incubation for 30 min at 37°C.

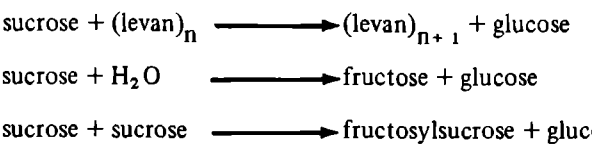
Influence of various cations and EDTA. The effect of various cations tested at a concentration of 5.10^{-3} M is shown in Table III. Both Ca^{2+} and Mg^{2+} were found to activate the enzyme; this effect could be abolished by the addition of an equimolar amount of EDTA. The enzyme was slightly activated in the presence of 0.02 M EDTA.

Table III Effect of cations on the activity of levansucrase from *A. viscosus*. The enzyme (0.04 U/ml) was incubated in a mixture containing 0.125 M sucrose, 0.05 M phosphate buffer pH 6.8 and various cations at a concentration of 5.10^{-3} M. The reducing sugars released were determined.

Cation	Relative activity
none	1.00
Ca^{2+}	1.50
Mg^{2+}	1.32
Mn^{2+}	1.00
Cd^{2+}	0.63
Co^{2+}	0.50
Cu^{2+}	0.30
Hg^{2+}	0.30
Zn^{2+}	0.00

Discussion

Levansucrase transfers the fructosyl residue from sucrose to the primary alcohol at the C-6 fructose present at the nonreducing end of a growing levan chain or present in sucrose, or in water. This can be described by the following three parallel reactions (Ebert and Schenk, 1968).



The enzyme is exocellular in *Strep. mutans* (Carlsson, 1970) and *B. subtilis* (Dedonder, 1966) and intracellular in *A. levanicum* (Ebert and Schenk, 1968).

In the present study the enzyme of *A. viscosus* was found to occur exocellularly. Krichevsky's (1969) failure to demonstrate levansucrase activity

in the supernatant of glucose-grown cells can be most likely explained by proteolytic breakdown of the enzyme or by the production of an inhibitor in stationary cultures as suggested by the present findings The occurrence of proteolytic enzymes in *A viscosus* was recently demonstrated by Freedman *et al* (1973)

The production of levansucrase by *A viscosus* was studied in continuous and batch cultures at various glucose concentrations In the medium containing 1% glucose a steep decrease in the enzyme activity was observed at a dilution rate of approximately 0 04 This decrease is accompanied by a rise in the level of non-utilized glucose, this suggesting a relationship between the enzyme synthesis and the external glucose level However, this relationship may be indirect and result from secundary effects exerted by glucose, such as the production of inhibitory substances or proteolytic enzymes and in the case of batch cultures by retardation of the growth The results obtained in batch cultures are in favor of this view The reaction products of levansucrase from *A viscosus* were glucose, fructose and fructan Paper chromatography revealed that oligosaccharides were almost absent in the reaction mixtures No attempt has been made to characterize the fructan but the opalescent bluish solution of the polysaccharide suggested a considerably high molecular weight which is typical for bacterial fructans

The effect of metal ions on the activity of levansucrase from various microorganisms is rather inconsistent While the enzyme from *A viscosus* was activated by Ca^{2+} and Mg^{2+}, the enzyme from *Strep mutans* (Carlsson, 1970) was unaffected and the enzyme from *Strep salivarius* (Garszczynski and Edwards, 1973) was even inhibited by Ca^{2+} and Mg^{2+}

EDTA on the other hand, had an inhibitory effect on both streptococcal levansucrase but not on the enzymes from *B subtilis* (Rapoport, 1965) and *A viscosus* Therefore, metal ions seem to be not essential for the activity of the last mentioned enzymes

References

Carlsson, J.: A levansucrase from *Streptococcus mutans*. Caries Res 4 97 113 (1970).

Da Costa, T. and Gibbons, R.J. Hydrolysis of levan by human plaque streptococci. Arch. oral Biol. **13** 609-617 (1968)

Dedonder, R. Levansucrase from *Bacillus subtilis*, in Methods in Enzymology, Vol. VIII. pag. 500-505 Academic Press London. 1966

Dische, Z. and Devi, A. A new colorimetric method for the determination of ketohexoses in the presence of aldoses, ketoheptoses and ketopentoses. Biochim. Biophys Acta **39** 140-144 (1960)

Dubois, M.; Gilles, K.A , Hamilton, J K., Rebers, P A and Smith, F Colorimetric method for determination of sugars and related substances. Anal. Chem. **28** 350-356 (1956)

Ebert, K.H. and Schenk, G. Mechanism of biopolymer growth. the formation of dextran and levan Adv Enzymol **30** 179-221 (1968).

Freedman, M.L., Valuzzo, T.A and Brown A T Localization of proteolytic enzyme in *Actinomyces viscosus* J. dent Res. **52** 88 (1973). Abstracts of the 51th IADR meeting Washington.

Gabriel, D. and Wang, S.-F. Determination of enzymatic activity in polyacrylamide gels. Anal. Biochem 27 545-554 (1969).

Garszczynski, S.M. and Edwards, J R Synthesis of a broth levan by a cell-bound levansucrase from *Streptococcus salivarius* (SS_2). Arch. oral Biol. **18** 239-251 (1973).

Gerencser, M.A. and Slack, J.M.: Identification of human strains of *Actinomyces viscosus*. Applied Microbiol **18** 80-87 (1969).

Hartree, E.F.: Determination of Protein A modification of the Lowry method that gives a linear photometric response. Anal. Biochem. **48** 422-427 (1972)

Hoeven, J.S. van der A slime producing microorganism in dental plaque of rats, selected by glucose feeding. Caries Res. **8** 193-210 (1974).

Hotz, P.; Guggenheim, B. and Schmid, R.. Carbohydrates in pooled dental plaque. Caries Res. **6**:103-121 (1972).

Houte, J. van and Jansen, H.M. Levan degradation by streptococci isolated from human dental plaque. Arch. oral Biol. **13**: 827-830 (1968).

Krichevsky, M.I., Howell, A. and Lim, S.: Levan formation by *Odontomyces viscosus*. J. dent. Res. **48** 938-942 (1969).

Llory, H.; Guillo, B. and Frank, R.M.. A cariogenic *Actinomyces viscosus* - a bacteriological and gnotobiotic study. Helv. Odont. Acta **15**: 134-138 (1971).

Manly, R.S. Liberfarb, R.. Cormier, A., and O'Brien, A.: Automated analysis of levan content in dental plaque, in Proceedings of the 1966 Technicon Symposium Automation in analytical chemistry, p. 254-256 (1966).

Manly, R.S and Richardson, D.T. Metabolism of levan by oral samples. J. dent Res **47** 1080-1086 (1968).

Maurer, H.R. Disk Elektrophorese Walter de Gruyter, Berlin, 1968.

McDougall, W.A. Studies on the dental plaque IV. Levans and the dental plaque Aust dent J **9** 1-5 (1964).

Mesner, Z. Formation of levanase by *Odontomyces viscosus* J. dent Res **50** 670 (1971).

Rapoport, G.. Composition en aminacides de la lévane-sucrase de *Bacillus subtilis*. Compt. Rend. Acad. Sci. **260** 1016-1019 (1965).

Rapoport, G. Propiétés générales et activetés enzymatiques de la lévane-sucrase purifiée de *Bacillus subtilis* Thesis (Paris 1966).

Rauen, H M. Biochemisches Taschenbuch II Springer Verlag, Berlin 1964, p 95

Somogyi, M.: A new reagent for determination of sugars J. biol. Chem **160** 61-73 (1945)

Snyder, M.L., Bullock, W W. and Parker, R.B. Morphology of Gram-positive filamentous bacteria identified in dental plaque by fluorescent antibody technique. Arch. oral Biol **12** 1269-1273 (1967).

Trevelyan, W.E. Procter, D.P and Harrison, J.S. Detection of sugars on paperchromatograms Nature (London) **166** 444-445 (1950).

Vesterberg, O. Separation of proteins from carrier ampholytes. Science Tools **16** 24-27 (1969)

Wood, J.M. Polysaccharide synthesis and utilization of dental plaque. J dent Res **43** 955 (1964)

Wood, J.M. and Critchley, P The extracellular polysaccharide produced from sucrose by a cariogenic streptococcus. Arch oral Biol **11** 1039-1042 (1966)

STELLINGEN

I

De tandheelkundige verzorging in Nederland is een voorbeeld van ongelijke verdeling van een schaars goed.

II

De normen die worden gehanteerd bij het 13-jarigenplan, dat bedoeld is om uitgebreide tandheelkundige hulp te verlenen aan bij het ziekenfonds aangesloten kinderen vanaf hun 13e jaar, sluiten het grootste deel van deze kinderen uit.

Staatscourant, 4 juli 1974

III

Er is geen reden om te veronderstellen dat glucose een geschikt vervangingsmiddel voor suiker is bij de bestrijding van tandbederf en tandvleesontsteking.

IV

Bij het onderzoek naar een verband tussen caries en antilichamen tegen cariogene streptokokken hebben Kennedy, Lehner en Berkenbildt ten onrechte geen rekening gehouden met kruisreakties van bakteriele antigenen.

Kennedy et al Archs oral Biol. 13, 1275-1278 (1968)
Lehner et al. Archs oral Biol. 15, 481-490 (1970)
Berkenbildt et al J Am. dent Ass 83, 332-337 (1971).

V

Bakterien in de tandplaque verkeren in een toestand van langzame groei en zijn daardoor gevoeliger voor remming door fluoride

VI

Het beheer van de Overasseltse en Hatertse vennen door Staatsbosbeheer heeft niet geleid tot bescherming van dit natuurgebied

VII

Veel bejaarde mensen bij wie een licht verminderde glucose tolerantie is vastgesteld, gebruiken nodeloos een suikervrij dieet

VIII

Een studie over Barbey d'Aurevilly had in het boek van Castex 'Le conte fantastique en France' niet mogen ontbreken

J S van der Hoeven

Nijmegen, 27 september 1974